THE EFFECTS OF CHRONIC ETHANOL EXPOSURE ON PRESYNAPTIC CHOLINERGIC RECEPTOR MODULATION OF ^{3}H-GABA RELEASE IN RAT HIPPOCAMPUS

By

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for my beautiful daughter,

Helen Y. Hu

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Chronic ethanol treatment (CET) for 28 weeks significantly increases electrically stimulated ^{3}H-GABA release from rat hippocampal slices. This increase in GABA release may be one of the mechanisms by which CET decreases the magnitude of long term potentiation (LTP) in the hippocampus. Evidence indicates that there is presynaptic cholinergic regulation of GABA neurotransmission, and it is possible that CET may alter this regulation. This study examined whether CET increases GABA release via an alteration in presynaptic cholinergic regulation.

We found that carbachol increased ^{3}H-GABA release in a concentration-dependent manner and atropine modulated ^{3}H-GABA release in a biphasic concentration-dependent manner. Atropine significantly blocked the effects of carbachol. Mecamylamine did not modulate ^{3}H-GABA release and did not block the

effects of carbachol. These results suggest that presynaptic muscarinic receptors facilitate GABA release, while nicotinic receptors do not play a significant role in modulating GABA release in hippocampus. The effects of these agents were also tested in rats 0 or 48 hrs after withdrawal from CET. The biphasic effects of atropine were decreased, whereas the facilitating effects of carbachol were significantly increased. There were no changes in the effects of these agents in modulating ^{3}H-acetylcholine release from hippocampal slices of CET rats compared to sucrose-treated rats. These results indicate that CET selectively alters presynaptic muscarinic regulation of GABA release in hippocampus.

We further measured ^{3}H-GABA release in the presence of selective agonists and antagonists for muscarinic receptor subtypes. We found that McNA-343, a selective M1 muscarinic receptor agonist, significantly decreased ^{3}H-GABA release while pirenzipine, a selective M1 muscarinic receptor antagonist, significantly increased ^{3}H-GABA release. On the other hand, oxotremorine, a selective M2 muscarinic receptor agonist, significantly increased ^{3}H-GABA release while AF-DX116, a selective M2 muscarinic receptor antagonist, significantly decreased ^{3}H-GABA release in a dose-dependent manner. Pirenzipine blocked the effects of McNA343 but not oxotremorine. AF-DX116 blocked the effects of oxotremorine but not McNA343. These results suggest that M1 muscarinic receptors inhibit GABA release, and M2 muscarinic receptors facilitate GABA release in rat hippocampus. The effects of these agents were also tested in rats 48 hrs after withdrawal from CET. We found that the effect of McNA343 was decreased whereas the effects of oxotremorine and AF-DX116 were significantly increased in CET rats. These results indicate that CET increases GABA release in hippocampus via selectively increase presynaptic M2 muscarinic receptor and inhibit M1muscarinic receptor function.

CHAPTER I
INTRODUCTION

Chronic alcohol abuse results in brain damage and persistent memory loss (Lynch, 1960; Victor et al., 1971; Butters, 1985). It has been shown that chronic ethanol treatment (CET) has a long-lasting effect on both learning and memory (Walker & Hunter, 1978). Evidence also indicates that CET diminishes the magnitude of LTP in the hippocampus and the effect can last as long as seven months after ethanol withdrawal (Tremwel & Hunter, 1994). Therefore, it has been suggested that the CET-induced decrement in LTP may be one of the mechanisms for long lasting learning and memory dysfunction after CET. The effect of CET on the induction of LTP appears to cause greater changes in inhibitory GABAergic neurotransmission (Tremwel, Hunter & Peris, 1994) than in glutamate transmission (Tremwel, Anderson & Hunter, 1994). It has also been shown that CET significantly increases electrically-stimulated ^{3}H-GABA release from hippocampal slices but does not affect postsynaptic GABA$_A$ receptor function (Tremwel, Hunter & Peris, 1994). One mechanism by which CET may increase GABA release seems to be via decreasing presynaptic GABA$_B$ receptor function (Peris et al.,1997). However, other presynaptic influences on GABA release may also be involved.

Many reports show the strong correlation between central cholinergic pathways and the processes of learning and memory (Arendt et al., 1989;

Drachman,1989; Jerusalinsky et al., 1997). Cholinergic innervation also seems to be important for mediation of events leading to LTP. The balance between cholinergic and GABAergic systems may determine hippocampal theta activity (Van der zee & Luiten, 1993) which has been shown to modulate LTP *in vivo* (Mott & Lewis, 1990). GABAergic neurons in CA1, CA3, and the dentate gyrus of the hippocampus express muscarinic cholinergic receptors (Van der zee & Luiten, 1993); and more specifically, GABAergic interneurons in the hippocampus express M2 muscarinic acetylcholine (ACh) receptors (Hajos et al., 1998).

Thus it is possible that cholinergic receptors may play important roles in the regulation of GABA release, and this regulation can be affected by CET. The purpose of this dissertation was to investigate cholinergic presynaptic receptor modulation of GABA released from hippocampal slices and how CET alters this modulation.

CHAPTER 2
LITERATURE REVIEW

Chronic ethanol abuse is associated with the disruption of neuronal and cognitive function (Victor et al., 1971; Freund 1973; Parsons 1977; Butters, 1985). The major symptoms can range from mild cognitive deficits (Phillips & Gregg, 1983; Butters, 1985) to Korsakoff's syndrome or alcoholic dementia characterized by a profound anterograde amnesia (Victor et al., 1971; Butters, 1985). In general, the ethanol induced learning and memory deficits are progressively more severe as the duration of chronic ethanol abuse is increased and apparently irreversible following ethanol cessation of sufficient duration (Freund & Walker, 1971; Arendt et al., 1989). However, it is still not clear what is the specific morphological or functional basis of the ethanol-induced learning and memory deficits. The ethanol-induced learning and memory deficit may occur due to ethanol neurotoxicity and nutritional deficiency following chronic ethanol abuse. Although malnutrition may contribute to this disruption of neuronal and cognitive function in man (Butters, 1985), it was also seen that brain damage and memory deficits associated with chronic ethanol abuse occurs in the absence of malnutrition (Freund, 1973; Smith et al., 1973). Therefore, it is still controversial what the relative contribution of nutritional deficiency and ethanol neurotoxicity is to the mnemonic deficit (Freund, 1973; Greenberg & Diamond, 1985).

Effects of Chronic Ethanol on Hippocampus

The hippocampal formation has an essential role in certain types of memory (Milner 1959; Olton et al. 1979; Squire 1986, Zola-Morgan & Squire 1990). Much evidence indicates that the hippocampus and its afferent innervation from the basal forebrain may play an important role in ethanol induced memory deficits. Animal studies confirm that CET causes abnormal morphology and function in the rodent hippocampus. The behavioral disfunction observed following CET in animal studies may be due to the fact that CET causes the loss of neurons in the rat hippocampus (Walker et al, 1971,1980,1981,1993). The neuronal cell loss in hippocampus associated with CET includes a 10-40% loss of principal cells (Walker et al., 1980, 1981; Walker & Hunter, 1987; Cadete-Leite et al., 1988a and b, 1989; Durand et al., 1989; Lescaudron et al., 1989) and interneurons (Lescaudron et al., 1986; Scheetz et al., 1987). The extent of the cell loss depends upon the duration of CET, the magnitude of exposure, genetic susceptibility to ethanol, and the length of ethanol abstinence.

Neurons which survive CET also exhibit structural abnormalities. These morphological changes are often region specific (Riley & Walker, 1978; Goldstein et al., 1983; McMullen et al., 1984; King et al., 1988). Surprisingly, direct electrophysiological studies show that CET produces only subtle changes in the function of the hippocampus. These functional changes include a reduction in intrinsic inhibitory processes (Abraham et al., 1981; Durand & Carlen, 1984a; Rogers & Hunter, 1992) and a modification in the distribution of synaptic connections using current-source density analysis (Abraham & Hunter, 1982; Abraham et al., 1982)

The Mechanism Underlying the Effects of CET on LTP

A more profound effect of CET on hippocampal function is that CET appears to alter the capacity for synaptic plasticity such as long-term potentiation (LTP). LTP is a form of synaptic potentiation usually elicited by brief high-frequency tetanic stimulation (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973). It is manifested by an increase in the amplitude and the slope of the extracellular recorded EPSP elicited by low-frequency stimulation of the synapse. LTP can persist for several hours in vitro and up to several weeks in vivo (Ben-Ari et al., 1992). There are several important correlations that support the hypothesis of a link between LTP and memory (Jerusalinsky et al., 1997) and although it is still controversial, LTP is generally considered to be a synaptic model or substrate for learning and memory (Teyler & Discenna, 1984; Bliss et al., 1988). It has been shown that CET has a long lasting effect on both learning and memory (Walker & Hunter, 1978). Evidence also indicates that CET diminishes the magnitude of LTP in the hippocampus and the effect can last as long as seven months after ethanol withdrawal (Tremwel & Hunter, 1994). Therefore, it has been suggested that the CET-induced decrement in LTP may be one of the mechanisms for long lasting learning and memory dysfunction after CET.

LTP is ordinarily divided into three separate processes: 1) induction, 2) maintenance, and 3) expression. At the physiological level, the induction of LTP requires cooperativity of synaptic inputs resulting in a threshold depolarization of the postsynaptic membrane. It is generally accepted that both activation of NMDA receptors (Collingridge & Lester, 1989) as well as inactivation of GABA receptors (Wigstrom & Gustafsson, 1985) are important in the generation and maintenance of LTP. The induction of LTP may be mediated by glutamate acting on AMPA and then NMDA receptors (Mulkey & Malenka, 1992). The maintenance of LTP may involve alterations in the release of numerous neurotransmitters (Nicoll & Malenka, 1995), including GABA.

The current view of the role of GABAergic interneurons in the hippocampus has shifted from one of merely dampening neuronal activity to that of an active role in information processing. Accumulating evidence also suggests that the role of GABAergic interneurons are important for setting the conditions for synaptic changes such as LTP in hippocampal principal neurons (i.e., granule cells and pyramidal neurons) during learning and memory (see Lynch, 1998). Presynaptic $GABA_B$ receptors are proposed as a mechanism affecting LTP since a decrease in GABA release may contribute to an increased NMDA response (Mott et al., 1990; Davies et al., 1991; Mott & Lewis, 1991) and $GABA_B$ receptors can control the release of GABA from the synaptic terminal. During induction of LTP, $GABA_A$-mediated inhibition is decreased thereby allowing NMDA-mediated excitation to increase (Mott et al., 1990). The effect of CET on the induction of LTP appears to cause greater changes in inhibitory GABAergic neurotransmission (Tremwel, Hunter & Peris, 1994) than in facilitory glutamate transmission (Tremwel, Anderson & Hunter, 1994). It has also been shown that CET significantly increases electrically-stimulated ^{3}H-GABA release from hippocampal slices but does not affect postsynaptic GABA$_A$ receptor function (Tremwel, Hunter & Peris, 1994). One mechanism by which CET may increase GABA release seems to be via decreasing presynaptic GABA$_B$ receptor function (Peris et al.,1997). However, other presynaptic influences on GABA release such as cholinergic influences may also be involved.

The Role of Cholinergic Receptors in Hippocampal LTP

Cholinergic innervation of the hippocampus is widespread, underlying the multi-faceted effects of ACh as a modulator of hippocampal transmission. There are many reports showing the strong correlation between central cholinergic pathways and the processes of learning and memory (Arendt et al., 1989; Drachman, 1989; Jerusalinsky et al., 1997). Cholinergic innervation also seems to be important for mediation of events leading to LTP. The balance between cholinergic and GABAergic systems may determine hippocampal theta activity (Van der zee & Luiten, 1993) which has been shown to modulate LTP in vivo (Walker et al., 1980). The dentate gyrus is innervated by GABAergic and cholinergic fibers from the medial septum and the nucleus of the diagonal band (Thompson 1994) which can influence dentate gyrus excitability. Septohippocampal lesions block the effects of ethanol on LTP (Steffensenn et al., 1993). Whether this effect is due to a loss of altered cholinergic influences on GABA neurons remains to be answered, since it also has been shown that there is a strong cholinergic influence on GABAergic and other neurons in hippocampus (Baisden et al.,1984; Leranth & Frotscher, 1987; Ferraro et al., 1997). GABAergic neurons in CA1, CA3 and dentate gyrus of hippocampus express muscarinic cholinergic receptors (Van der zee & Luiten, 1993); and more specifically, GABAergic interneurons in hippocampus express M2 muscarinic ACh receptors (Hajos et al., 1998).

It has been reported that muscarinic receptors modulate GABA release in different brain regions. For example, muscarinic receptors stimulate GABA release in substantia gelatinosa of the rat spinal dorsal horn (Baba et al., 1998). In rat striatal nerve terminals, muscarinic receptors inhibit GABA release. It has been reported that there are a variety of effects of muscarinic receptors on GABAergic nerve terminals depending on different subtype and brain location. M1 muscarinic receptors are involved in modulation of GABA release in different brain regions. M1 muscarinic receptors mediate inhibition of GABA release in rat cerebral cortex (Hashimoto et al., 1994), but facilitate GABA release in neostriatum (Harsing & Zigmond 1998). M2 muscarinic receptors have been reported to mediate stimulatory effects on GABA release in the rat substantia nigra (Kayadjanian et al., 1994).

Cholinergic influences also appear to be involved in the effects of CET on LTP in hippocampus. There are numerous reports that suggest that CET may induce a permanent loss in cholinergic function in hippocampus. CET decreases high affinity choline uptake in hippocampus (Beracochea et al., 1986) as well as ACh levels, choline acetyltransferase and acetylcholinesterase activity (Arendt, 1988a & b, 1989). Lesions of the septohippocampal nucleus block the effects of acute ethanol on LTP (Steffensenn et al., 1993). Whether this effect is due to a loss of altered cholinergic influences on GABA neurons remains to be answered.

The Effects of CET on $GABA_A$ Receptor Composition

CET may affect postsynaptic $GABA_A$ receptors. The $GABA_A$ receptor is a multimeric membrane-spanning ligand-gated chloride channel, that admits chloride upon binding of the neurotransmitter GABA and is modulated by many endogenous and therapeutically important agents. It is a hetero-oligomer composed of a total of 5 polypeptide subunits of at least seventeen genetically distinct types (α_{1-6}, β_{1-4}, γ_{1-4} , δ, ρ_{1-2}) and alternatively spliced variants, each displaying a unique regional expression (Laurie et al., 1992; Wisden et al., 1992). The composition of $GABA_A$ receptor subunits determine the sensitivity of the receptor complex to different compounds including ethanol. The γ_2 subunit is necessary for benzodiazepine modulation of function (Pritchett et al., 1989) and the alternatively spliced γ_{2L} form of this subunit must be appropriately phosphorylated by protein kinase C before ethanol sensitivity is conferred (Wafford et al., 1991; Aguayo & Pancetti, 1994; Weiner et al., 1994). In receptors that are sensitive to ethanol, there is an enhancement of GABA-stimulated chloride ion (Cl) conductance (Sanna & Harris, 1993). Subchronic ethanol exposure (5-10 days) results in a loss of in vitro enhancement of channel function by ethanol (Allan & Harris, 1987; Morrow et al., 1988). Although there has not been any consistent evidence for a change in the number or affinity of the $GABA_A$ receptor as a whole (Thyagarajan & Ticku, 1985;

Tamborska & Marangos, 1986), subchronic ethanol exposure decreases both mRNA and peptide levels for α_1, α_2 and α_3 subunits in cortex, increases α_6 in cerebellum (Morrow et al., 1990; Buck et al., 1991; Montpied et al., 1991; Mhatre & Ticku, 1992; Morrow et al., 1992; Mhatre et al., 1993) and causes long-term increases in both mRNA and peptides for the β_2 and β_3 subunits in both regions (Mhatre & Ticku, 1994). Thus, subchronic ethanol exposure changes the subunit composition and very likely, the functional status of the receptor. However, most of the changes described above are transient in nature, returning back to control levels within 48 hrs after ethanol withdrawal. Whether similar changes occur in hippocampus in response to CET sufficient to cause permanent changes in LTP is not known.

Both subchronic ethanol exposure and CET decrease GABA immunocytochemistry in CA1 (Lescaudron et al., 1986; Fifkova et al., 1994) which may indicate a loss of inhibitory interneurons. This loss may be the probable basis for CET disruption of synaptic inhibition in CA1 (Rogers & Hunter, 1992) and CET induced decrease in spontaneous IPSPs (Durand & Carlen, 1984a). The CET-induced loss of GABA neurons appears to be more pronounced in ventral hippocampus compared to dorsal regions (Lescaudron et al., 1986) which may be due to greater cholinergic innervation of this area. Our studies indicate that the interactions of cholinergic receptors and GABA release may be important for mediating the effects of CET on LTP.

Classification of Muscarinic Receptor Subtypes

So far at least five muscarinic receptor subtype genes (m1, m2, m3, m4, and m5) have been cloned (Bonner et al., 1987) and are expressed in the hippocampus to varying degrees (Li et al., 1991; Levey, 1993). Operational criteria have suggested the presence of at least four subtypes in nature, they are denoted as M1, M2, M3, and M4 (Hulme et al., 1990). A physiological role for the m5 gene product remains to be identified (Hosey 1992). All of these receptors are related structurally to receptors which are known to activate guanine nucleotide-binding regulatory proteins, or G-proteins (Honkanen & Abdel-latif, 1989; Avissar & Schreiber, 1989; Hosey, 1992). M1, M3 and M5 muscarinic receptors couple to stimulate phospholipase C, while M2 and M4 muscarinic receptors inhibit adenylyl cyclase. The pharmacological profile of each muscarinic receptor subtypes is ambiguous, mainly because many tissue and cell lines express heterogenous populations of muscarinic receptor subtypes (Hulme et al. 1990) and due to the lack of good selective agents for the muscarinic receptor subtypes.

Muscarinic receptor agonists alone are not generally useful in discriminating muscarinic receptor subtypes, since no agonists can discriminate between muscarinic receptor subtypes on an affinity basis. More definitive as tools in muscarinic receptor subtype classification are antagonists. In general, muscarinic M1 receptors are pharmacologically defined by a high affinity toward pirenzipine and 4-DAMP and a low affinity for methoctramine or himbacine. The muscarinic M2 receptors, in contrast, are pharmacologically defined by a high affinity toward tripitramine, methoctramine or himbacine and a low affinity for pirenzipine and 4-DAMP (see Eglen & Walson, 1996).

Several agonists and antagonists have been identified that exhibit a functional selectivity for muscarinic M1 and M2 receptors (Moltzen & Bjornholm 1995). These compounds are so termed since they activate one muscarinic receptor subtype preferentially by virtue of the prevailing high receptor subtype reserve rather than differential affinity. For example, McN-A-343, frequently cited as a selective muscarinic M1 receptor agonist, exhibits a potency contingent upon the prevailing muscarinic M1 receptor reserve in hippocampus (Black & Shankley 1985; Eglen et al. 1987). Oxotremorine, a muscarinic agonist, has two binding sites with Kd values of 1.2 nM and 446 nM (Miyoshi et al. 1986). Oxotremorine selectively acts at the muscarinic M2 receptor via the high-affinity binding site (Miyoshi et al. 1986). AF-DX 116 is an antagonist with a relatively high affinity for the muscarinic M2 receptors and modest affinity for the muscarinic M4 receptor (Schulte et al. 1991).

In summary, muscarinic receptor subtypes, when defined pharmacologically, correspond with those identified on the basis of sequence. Several antagonists are now availably that, when used in concert with agonists, allow reasonably good definition of subtypes.

Effects of Muscarinic Receptor Subtypes in Hippocampus

Anatomical and physiological evidence suggests that ACh, acting via muscarinic receptors, can differentially modulate excitatory and inhibitory transmission at many sites along the hippocampal circuits. Earlier studies with the classical antagonist quinuclidinyl benzilate (QNB) suggested a homologous population of binding sites for brain muscarinic receptors. Subsequently, it has been shown that selective agonist and antagonists can distinguish binding sites with low-, high-, and super high-affinity (Birdsall et al 1978; Hulme et al., 1983). Using the antagonist pirenzipine, Hammer et al. (1980) distinguished two subclasses of muscarinic receptors, M1 and M2. Autoradiographic studies show different localization of M1 and M2 receptors in the brain. Seventy to ninety percent of muscarinic receptors in the cerebral cortex, hippocampus and striatum are M1 receptors, whereas those in the pons and medulla oblongata are predominantly M2 receptors (Potter et al., 1984; Messer & Hoss 1987). Generally, M1 receptors are located postsynaptically mainly on dendritic tufts of neurons (Vogt & Burns 1988), and M2 are located presynaptically on axon terminals (Marchi and Raiteri 1985; Vogt & Burns 1988). M2 receptors are also located postsynaptically on both dendritic tufts and axon terminals of GABAergic interneuron in hippocampus (Hajos et al., 1998).

The Effects of Chronic Ethanol on Muscarinic Receptors

Ethanol has been shown to have both acute and chronic effects on the function of several receptor systems. Some of these effects may underlie ethanol's acute and chronic effects on neuropsychological function and ethanol-induced brain damage. CET usually leads to regionally selective increases in muscarinic receptor density. Ethanol does not affect the binding of ligand to brain muscarinic receptors in vitro (Pietrzak et al., 1988). Similarly, acute treatment of animals with ethanol has no effect on the binding of ligand to these receptors (Wigell & Overstreet 1984). The use of selective ligands to differentiate muscarinic receptor subtypes reveals the effects of ethanol to be regionally selective. Pietrzak et al., (1989) assayed muscarinic receptors in rats treated continuously with ethanol for up to 2 years. At 3 and 9 months there was an increased density of cortical muscarinic receptors. In hippocampus, 21 months of exposure was required to produce significant change in receptors. In general, the results of animal studies suggested that the selective increases in muscarinic receptor density by CET was greatly influenced by the strain of animal, method of ethanol administration, and duration of treatment (Shanly & Wilce, 1993).

CET damages the septohippocampal projection resulting in loss of basal forebrain neurons, which is paralleled by a decline in ACh, choline acetyltransferase and acetylcholinesterase in the forebrain and hippocampus (Arendt et al., 1989). Losses of chemically specific afferents, including cholinergic afferents, often elicit postsynaptic changes which appear to at least partially compensate for the loss of input, i.e. receptor supersensitivity (Bird & Aghajanian, 1975). CET, however, results in structural as well as biochemical damage to surviving hippocampal neurons (Walker et al., 1993a,1993b). It has therefore been unclear whether the damage to the septohippocampal cholinergic projection produced by CET is accompanied by corresponding changes in cholinergic responses in surviving hippocampal neurons. CET decreases the effects of ACh on population spike amplitude but has no effects on carbachol-inhibition of EPSPs (Rothberg & Hunter, 1991). Additionally, both cholinergic disinhibition and recurrent inhibition are decreased by CET. These data imply that CET may decrease the number or functional status of muscarinic cholinergic receptors in hippocampus. Evidence indicates this is not due to an effect of CET on muscarinic subtype densities (Rothberg et al., 1993; Rothberg et al., 1996). The inhibitory effect of carbachol on the EPSP is thought to be due to the presence of presynaptic muscarinic cholinergic receptors on the terminals of the stratum radiatum afferents. When these receptors are activated, neurotransmitter release is reduced, thus, the effects of CET seem to be selective for postsynaptic rather than presynaptic cholinergic receptors. It is possible that cholinergic receptors may play important roles in regulation of GABA release in hippocampus, and this regulation can be affected by CET.

Objectives of the Dissertation

The role of GABAergic interneurons in cortical network function has shifted from one of merely dampening neuronal activity to that of an active role in information processing (Paulsen & Moser, 1998). The neuronal network in hippocampus is complicated. it has been suggested that principle cells (that is, granule cells or pyramidal cells) contacted by several neurons such as, GABAergic interneurons, glutamatergic neurons and cholinergic input via feed-forward as well as feedback circuits. Furthermore, GABAergic interneurons are also innervated by cholinergic input. However, it is still not clear the relationships between these neurons and how CET selectively alters these relationships. Before discussing the results from these studies, it is important to describe this complicated neuronal network as a simplified system (Figure 1-1). In this model, we hypothesized that principle cells are innervated by both inhibitory GABAergic interneurons and stimulatory glutamate input. The balance between these two neurotransmitters determine the activity of principle cells. GABAergic interneurons are innervated by cholinergic input via inhibitory M1 muscarinic receptors and stimulatory M2 muscarinic receptors. The activity of GABAergic interneurons is determined by the balance of M1 and M2 muscarinic receptors.

The major objective of this dissertation was to discover the mechanisms that might underlie the decrement in LTP resulting from ethanol treatment. The main hypothesis tested by the experiments presented in this dissertation is that long term changes in GABAergic and cholinergic neurotransmitter systems in hippocampus occur, which could explain the inhibition of LTP by CET. Specifically, there are long-term increases in GABAergic neurotransmitter release caused by CET. We further hypothesized that different muscarinic receptor subtypes located on GABA interneurons modulate GABA release in a complex manner and CET can selectively alter this muscarinic regulation of GABA release. This persistent increase in GABA release is due to down regulation of inhibitory muscarinic receptors and up regulation of facilitory muscarinic receptors. In the present study, we measured the amount of ^{3}H-GABA released from hippocampal slices in response to high frequency stimulation and the modulation of this release by cholinergic presynaptic receptors. The effect of CET on this modulation was also determined. In general, our data show that presynaptic muscarinic cholinergic receptors facilitate ^{3}H-GABA release in hippocampal slices, and CET increased this facilitating muscarinic cholinergic modulation of GABA release. These results are consistent with increased GABA release after CET. There were no effects of CET on cholinergic modulation of ACh release. These results may help us to elucidate the mechanisms underlying the disruption of LTP by CET.

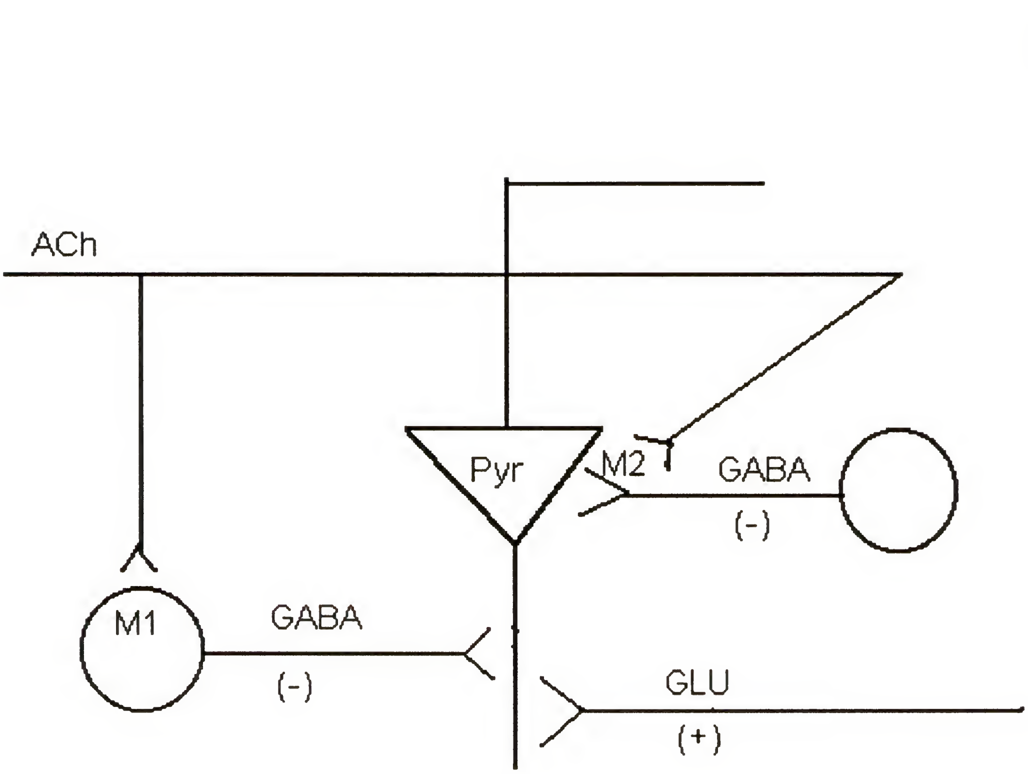

Figure1-1. Schematic diagram of hypothesized neuronal network in hippocampus. Principle cells (that is, granule cells or pyramidal cells, shown as triangle) are contacted by inhibitory GABAergic interneurons (shown as circle) and facilitory glutamatergic input. GABAergic interneurons are innervated by cholinergic input. ACh inhibits the activity of GABAergic interneurons via M1 muscarinic receptors and facilitates GABAergic interneurons via M2 muscarinic.

CHAPTER 3
GENERAL METHODS

Adult male Sprague-Dawley and Long-Evans hooded rats (225-250g; Charles River Breeding Laboratories) were housed one per cage in a 24°C controlled environment with a 7:00 A.M. to 7:00 P.M. light cycle . These rats were used to characterize cholinergic modulation of ^{3}H-GABA release in naive animals.

CET rats were treated as described previously (Tremwel, Anderson & Hunter, 1994). Briefly, male Long-Evans rats (200-250 g; Charles River) were paired by weight and assigned to one of two liquid diet treatment groups: 1) Group E received diet in which ethanol comprised 35-39% of the total caloric intake (8.1 -9.4% v/v ethanol); 2) Group S received an identical diet except sucrose was isocalorically substituted for ethanol. The remaining calories were supplied via Sustacal (Mead-Johnson Co.) fortified with several times the daily requirement of essential vitamins and nutrients (Walker et al., 1980; Walker & Freund, 1971).

The amount of ethanol diet consumed per Group E animal each day was measured and an equal volume of sucrose diet was given on the following day to the pair-fed Group S animal. The liquid diets were freshly prepared and administered daily between 8:00 and 9:00 A.M. as the sole source of food or liquid for 28 weeks. The percentage of calories in the form of ethanol or sucrose was increased from the initial level of 35% total caloric intake by 1% every four weeks until 39% was achieved. The average daily intake of ethanol using this paradigm was 12-13 g/kg/day. At the end of the 28-week period, all animals received laboratory chow and water *ad libitum* for 0 or 48 hours after which they were killed by decapitation without anesthesia and tissue samples were obtained.

^{3}H-GABA release and ^{3}H-ACH release were measured using an adaptation of a method described previously (Peris et al., 1992, 1997). The hippocampus was dissected from both sides of a single rat brain and 0.4 mm thick transverse slices were cut using a Brinkmann tissue chopper. These slices were incubated in Krebs' buffer (pH = 7.4) saturated with 95% O_2/5% CO_2 for 30 min at 34°C. The composition of the Krebs' buffer was (in mM): NaCl, 118; glucose, 11.1; $NaHCO_3$, 25; KCl, 4.7; NaH_2PO_4, 1.0; $MgCl_2$, 1.2; $CaCl_2$, 1.3; EDTA, 0.004. For ^{3}H-GABA release, 0.1 mM aminooxyacetic acid (a GABA-transaminase inhibitor) was added. The medium was replaced with 4 ml fresh buffer containing 10 µl of ^{3}H-GABA (80 Ci/mmol; final concentration about 32 nM). For ^{3}H-ACh release, 0.1 mM physostigmine (an ACh-esterase inhibitor) was added to the Krebs' buffer. The medium was replaced with 4 ml fresh buffer containing 10 µl of ^{3}H-choline (85 Ci/mmol; final concentration about 30 nM). The incubation was continued for 30 min. Rinsed, prelabeled slices were then placed into separate glass chambers maintained at 34 °C and perfused with oxygenated Krebs' buffer at 1 ml/min.

The superfusate was collected for 95 min at 5 min intervals beginning 60 min after the start of superfusion. Slices were exposed to 120 sec of electrical field stimulation (unipolar square wave pulses, 2 msec duration, 30 Hz, 8-30 mA) at 20

(S1) and 65 (S2) min after the start of collection. Superfusion with medium containing different concentrations of receptor agonists and antagonists was started 20 min after S1 (45 min after the start of collection) and continued at a constant level until slices were removed from the chambers. In some experiments, receptor antagonists were present throughout superfusion and agonist was added 20 min after S1. Previous experiments have shown 10-15 min superfusion with different solutions is sufficient to establish stabilized effects on release (Van der zee & Luiten, 1993). Each concentration was tested on duplicate slices; three control slices (never exposed to drug) were included in each experiment. After superfusion, slices were solubilized (TS1, Research Products Inc.) and radioactivity in tissue and superfusate samples was determined by liquid scintillation counting.

The amount of tritium released in each fraction was expressed as a percentage of the total tritium content of each slice at the time of sample collection. Stimulated tritium release was calculated from the sum of all fractions greater than basal release occurring before stimulus onset. The effect of *in vitro* drug exposure was estimated by comparing S2/S1 ratios for control and treated slices.

Two-way ANOVA was used for all parametric analyses followed by Dunnett's multiple comparisons. The distribution of S2/S1 data is normal. Data is represented as mean±SEM with N equal to number of rats.

CHAPTER 4

THE EFFECT OF ELECTRICAL STIMULATION AND Ca^{2+} ON GABA RELEASE FROM RAT HIPPOCAMPAL SLICES

Introduction

An important criterion for the establishment of the neurotransmitter role for a substance is that it should be released from nerve terminals as a result of presynaptic stimulation. Therefore, depolarization of neurons in superfused brain slices by electrical stimulation or by perfusion with a medium containing a high potassium concentration (> 40 mM) are ideal methods for studying GABA release from nerve terminals. These procedures caused a striking increase in the efflux of ^{3}H-GABA from brain slices (Srinivasan, et al., 1969). The amount of ^{3}H-GABA release is dependent on the kind of stimulation. Electrical stimulation of brain slices with rectangular pulses (duration, 5 msec; frequency 100/sec; current 20mA) for 30 sec produced increased ^{3}H-GABA release in cortex to a maximum of 3.3 times the prestimulation value (Srinivasan, et al., 1969).

The nature of the applied electrical stimulation is important for ^{3}H-GABA release. Experiments using sine wave stimulation failed to increase the ^{3}H-GABA release appreciably even when the stimulating current was raised to 50 mA. On the other hand, stimulation with rectangular pulses at frequencies above 50/sec was successful in releasing ^{3}H-GABA. Many experiments have demonstrated the critical stimulation conditions, in which frequency ranges from 10 to 60/sec and stimulation time is above 60 sec, to evoke reliable efflux of ^{3}H-GABA from different brain region. Electrical stimulation increases several-fold the release of GABA from different brain areas (Oja et al., 1977). Previously, we used rectangular pulses (30 HZ frequency for 120 sec) of electrical stimulation to evoke ^{3}H-GABA release from hippocampal slices. These parameters can evoke a 2-4 fold increase in ^{3}H-GABA release from rat hippocampal slices (Peris et al., 1997). However, it is still necessary to establish the ideal parameters that can evoke the most reliable ^{3}H-GABA release from rat hippocampal slices.

The release of transmitters due to depolarization is believed to be mediated by a voltage-dependent increase in the influx of extracellular Ca^{2+} (Hubbard, 1970; Krnjevic, 1974; Blaustein, 1975). The dependence of release of GABA on extracelluar Ca^{2+}, however, has not been uniformly observed in brain hippocampal slices (Krnjevic, 1974). The effects of omitting Ca^{2+} from extracellular fluid depended on the nature of the releasing stimulus. With regard to the type of electrical pulses used, it has been reported that rectangular pulses, not sine-wave, evoke a Ca^{2+}-dependent ^{3}H-GABA release (see Saransaari and Oja, 1992). Ca^{2+} ions are considered a prerequisite for exocytotic emptying of storage granules of transmitters into synaptic clefts. On the other hand, rectangular pulses also evoke a Ca^{2+}-independent release of GABA from brain slices (Valdes & Orrego, 1978). This Ca^{2+}-independent stimulation-evoked release of GABA may originate in the cytoplasm and is dependent on Na^+ (Vizi, 1984).

The release of exogenous, labeled GABA from brain slices induced by electrical stimulation is not completely reduced by omitting Ca^{2+} (Katz et al., 1969; Srinivasan et al.,1969; Okada & Hassler,1973; Minchin & Nordmann, 1975; Miranda, 1976). However, the release of exogenous, labeled GABA from brain slices evoked by elevated K^+ from brain slices was consistently reduced in the absence of Ca^{2+} (Srinivasan et al., 1969; Mulder & Snyder, 1974; Reubi et al., 1977). It is still controversial whether the GABA release comes directly from cytoplasmic pools triggered by Na^+ elevation or from vesicular stores triggered by a Ca^{2+}-dependent exocytotic mechanism. Therefore, it is important to determine the effects of Ca^{2+} on GABA release in the current experiments.

The purpose of these experiments was: (1) To establish the electrical stimulation parameters that will elicit the largest and most reproducible signal. This was accomplished by measuring radio-labeled ^{3}H-GABA release from hippocampal slices exposed to 10, 20 or 30 Hz electrical pulses for 1, 2 or 3 min respectively. (2) To clarify the relationship between Ca^{2+}-dependent and independent release of exogenously labeled GABA induced by electrical stimulation in rat hippocampal slices. This was accomplished by measuring ^{3}H-GABA release from hippocampal slices after omitting calcium from the Krebs' buffer.

Methods

Animals

Adult male Sprague-Dawley (225-250 g) were obtained from Charles River Breeding Laboratories. Animals were housed one per cage in a controlled environment with a 7:00 A.M. to 7:00 P.M. light cycle and temperature of 24 °C. These rats were randomly assigned to different experiment groups in which some groups were used to test different electrical stimulation conditions and some groups were used to test the effects of Ca^{2+} on ^{3}H-GABA release.

Slice superfusion

^{3}H-GABA release were measured using the method described in chapter 3. Slices were exposed to 10, 20 or 30 Hz electrical pulses (unipolar square wave pulses 2 msec in duration, 8-30 mA) for 1, 2 or 3 min respectively at 20 (S1) and 65 (S2) min after the start of collection. After superfusion, the slices were solubilized (TS1, Research Products Inc.), and the radioactivity in both tissue and superfusate samples was determined by liquid scintillation counting. The amount of tritium released in each fraction was expressed as a percentage of the total tritium content of each slice at the time of sample collection. Stimulated tritium release was calculated from the sum of all fractions greater than basal release occurring before stimulus onset. The effect of different electrical stimulation conditions was estimated by comparing S1, S2, and S2/S1 ratios for control and treated slices.

Calcium withdrawal

The hippocampal slices were superfused with normal Krebs buffer with 1.2 mM $CaCl_2$. In some slices, Ca^{2+} free buffer was substituted 20 min before S2. Each concentration was tested on at least duplicate slices; at least three control slices (never exposed to drug) were included in each experiment.

Results

The Effect of Electrical Stimulation on ^{3}H-GABA release

As found previously (Peris et al.,1997; Tremwel, Hunter & Peris, 1994), basal ^{3}H-GABA release from hippocampal slices was stable over the 90 min superfusion period regardless of treatments. This was indicated by the fact that the basal release measured before S2 (PS2) was about 75% of that measured before S1 (PS1) (Table 4-1). The total tissue tritium was not changed regardless of different simulation treatment (Table 4-2).

To establish the electrical stimulation parameters that elicited the largest and most reproducible signal, we exposed hippocampal slices to 10, 20 or 30 Hz electrical pulses for 1, 2 or 3 min respectively. When hippocampal slices were exposed to rectangular pulses for 2 min, the electrically stimulated ^{3}H-GABA release from hippocampal slices was frequency dependent (Figure 4-1). The 10 Hz pulses for 2 min (1200 pulses) elicited the least amounts of tritium release measured as the smallest S1 and S2 value (Figure 4-1). The 30 Hz pulses for 2 min (3600 pulses) elicited the largest amount of tritium release (Figure 4-1). Although 10 Hz pulses for 2 min (1200 pulses) elicited the largest S2/S1 ratio (Table 4-3), 30 Hz pulse for 2 min (3600 pulses) elicited the most reliable S2/S1 ratio compared to that from 10 Hz pulse for 2 min or 20 Hz pulse for 2 min (Table 4-3). Considering S1, S2 and S2/S1 values, it appeared that 30 Hz pulses for 2 min elicited the largest and most reliable amounts of tritium release. ANOVA showed a significant main effect of frequency ($F(2,14) = 5.5$; $p < 0.05$).

The electrically stimulated ^{3}H-GABA release from hippocampal slices was time dependent (Figure 4-2). In these experiments, stimulation frequency was 30 Hz. 30 Hz electrical pulses for 1 min (1800 pulses) elicited the least amount of tritium release in either S1 or S2, and 30 Hz electrical stimulation for 3 min (5400 pulses) elicited the largest amount of tritium release. The 30 Hz electrical stimulation for 2 min (3600 pulses) also elicited a larger amount of tritium release (Figure 4-2) but the S2/S1 was larger and more reliable for 30 Hz electrical stimulation for 2 min compared to that from 30 Hz electrical stimulation for 1 or 3 min stimulation (Table 4-3). Considering S1, S2 and S2/S1 values, it appeared that 30 Hz pulses for 2 min (3600 pulses) elicited the largest and most reliable amounts of tritium release. One-factor ANOVA showed a significant main effect of stimulation time ($F(2,12)=8.2$; $p < 0.05$) on GABA release.

The electrically stimulated ^{3}H-GABA release from hippocampal slices was also pulse dependent (Figure 4-3). The 5400 pulses (30 Hz electrical stimulation for 3 min) elicited the largest amount of tritium release measured as the largest S1 and S2 value. The 3600 pulses (30 Hz electrical stimulation for 2 min) also elicited the larger amount of tritium release measured as the larger S1 and S2 values (Figure 4-3). But the S2/S1 is larger and more reliable for 3600 pulses (Table 4-3). Considering S1, S2 and S2/S1 values, it appeared that 3600 pulses (30 Hz pulses for 2 min) elicited the largest and most reliable amounts of tritium release. One-factor ANOVA showed a significant main effect of stimulation time ($F(2,18)=10.2$; $p < 0.05$).

Table 4-1. The basal ^{3}H-GABA release in hippocampal slices of adult rats was not affected by different electrical stimulation conditions. Basal release was measured before slices were exposed to each electrical field stimulation (PS1 and PS2). The values shown are mean ± SEM of PS2/PS1 ratio (n=5-10 for each group).

PS2/PS1 Ratios

	10 Hz	20 Hz	30 Hz
1 min			0.79±0.03
2 min	0.74±0.01	0.75±0.02	0.71±0.02
3 min			0.77±0.01

Table 4-2. The total ^{3}H-GABA in hippocampal slices of adult rats was not affected by different electrical stimulation condition. After superfusion, the slices were solubilized, and total ^{3}H-GABA was determined by calculating the ^{3}H-GABA in both tissue and superfusate samples. The values shown are mean ± SEM of total ^{3}H-GABA (n=5-10 for each group).

Total ^{3}H-GABA

	10 Hz	20 Hz	30 Hz
1 min			129872±13016
2 min	112560±9814	98421±7684	132411±10139
3 min			121987±13245

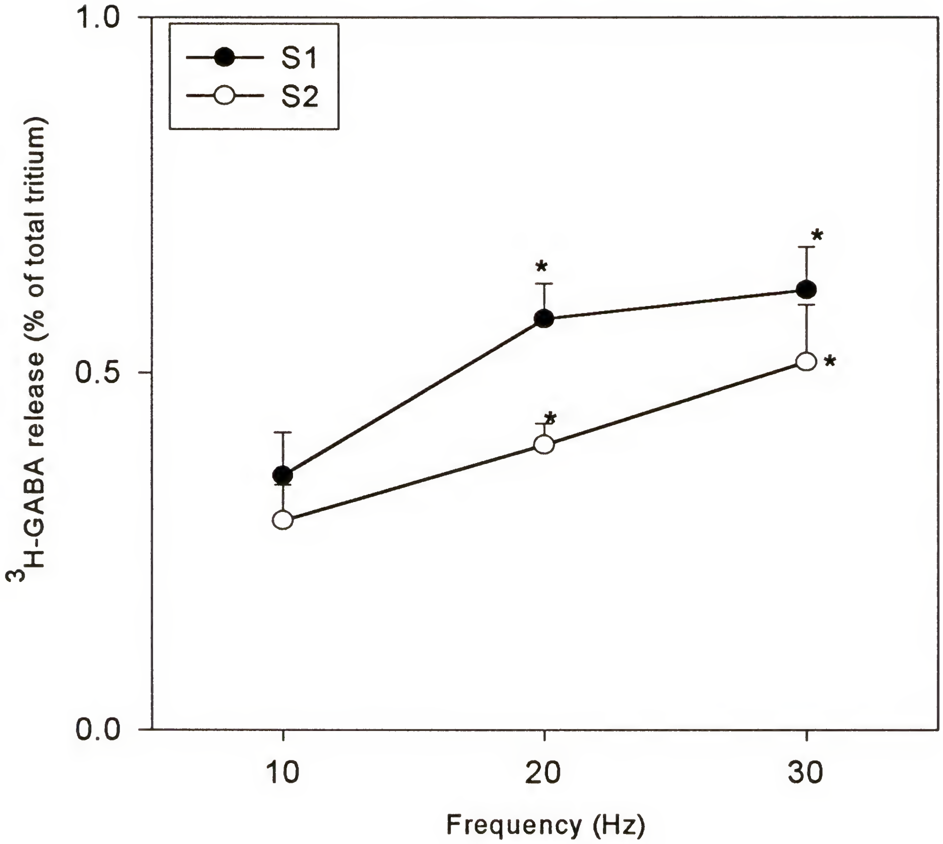

Figure 4-1. Electrically-stimulated ^{3}H-GABA release in hippocampal slices of adult Sprague-Dawley rats was tested under various stimulation conditions. The slices were exposed to 10, 20 or 30 Hz electrical pulses for 2 min at 20 (S1) and 65 (S2) min after the start of collection. The data are expressed as % total tritium in each slice. The values shown are means ± SEM with n=4-10. * $p < 0.05$ compared to each other condition.

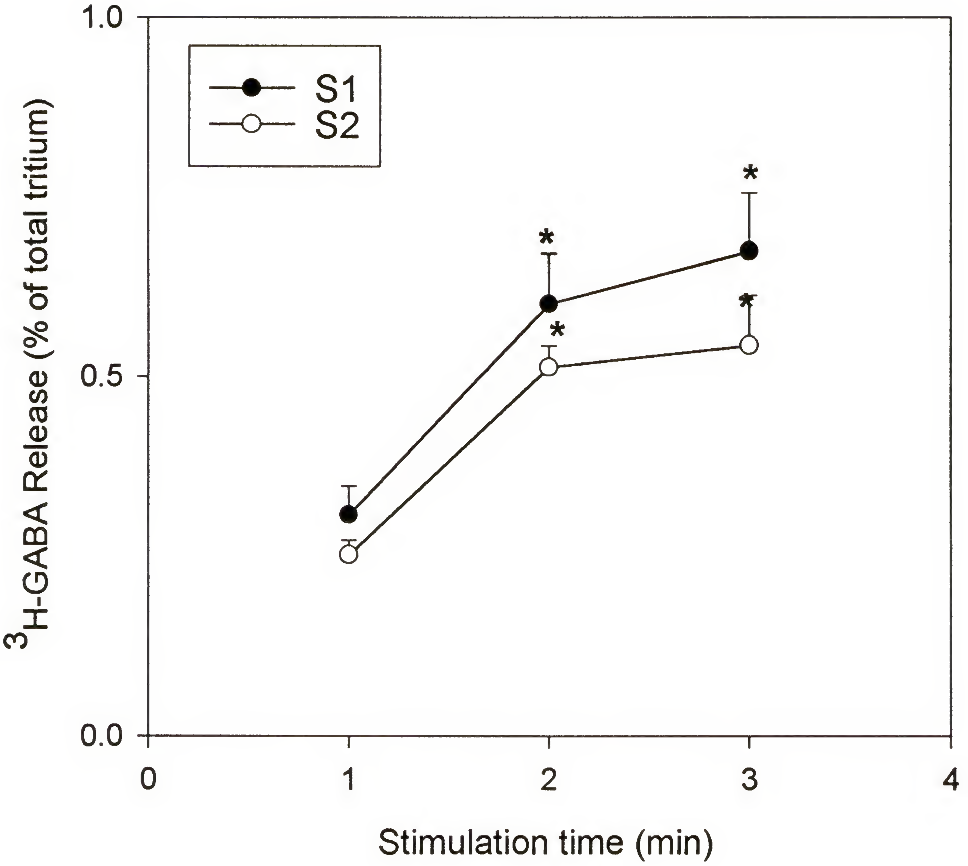

Figure 4-2. Electrically-stimulated ^{3}H-GABA release in hippocampal slices of adult Sprague-Dawley rats was tested under various stimulation conditions. The slices were exposed to 30 Hz electrical pulses for 1, 2 or 3 min at 20 (S1) and 65 (S2) min after the start of collection. The data are expressed as % total tritium in each slice. The values shown are means ± SEM with n=4-10 * $p < 0.05$ compared for each condition.

Table 4-3. Electrically stimulated ^{3}H-GABA release in hippocampal slices of adult rats was affected by different electrical stimulation conditions. Stimulated release was measured when slices were exposed to each electrical field stimulation (S1 and S2). The values shown are mean ± SEM of S2/S1 ratio (n=5-10 for each group).

S2/S1 Ratios

	10 Hz	20 Hz	30 Hz
1 min			0.81±0.05
2 min	0.79±0.06	0.65±0.06	0.75±0.03
3 min			0.67±0.04

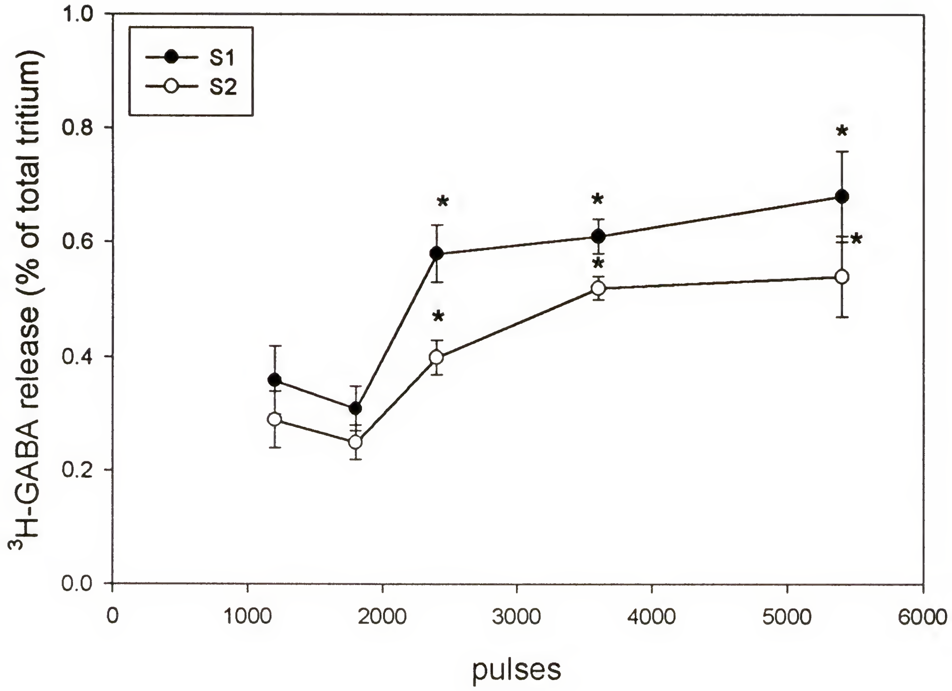

Figure 4-3. Electrically-stimulated ^{3}H-GABA release in hippocampal slices of adult Sprague-Dawley rats was tested under various stimulation conditions. The slices were exposed different electrical pulses at 20 (S1) and 65 (S2) min after the start of collection. The data are expressed as % total tritium in each slice. The values shown are means ± SEM with n=4-10 * $p< 0.05$ compared for each condition.

Ca^{2+} -Dependent and Independent GABA Release

I next tested whether electrically-stimulated ^3H-GABA release was calcium dependent by withdrawing calcium from the buffer 20 min before S2. ^3H-GABA release was not completely abolished by omitting Ca^{2+} from the buffer (Figure 4-4). Basal release of ^3H-GABA was significantly increased by 259% (Figure 4-5) as measured by the PS2/PS1 ratio. One-factor ANOVA showed a significant main effect of calcium ($F(4,7) = 10.7$; $p < 0.05$). Electrically stimulated ^3H-GABA release was significantly decreased but not completely eliminated by calcium withdrawal, with 56.9%±7% of ^3H-GABA release occurring in the absence of calcium compared to when calcium was kept at 1.2 mM (Figure 4-6). One-factor ANOVA showed a significant main effect of calcium ($F(4,7) = 8.7$; $p < 0.05$). This steep increase in basal ^3H-GABA release, complicates the interpretation of the second exposure to electrical stimulation. While the evoked ^3H-GABA release is decreased for S2, one cannot rule out the possibility of a ceiling effect on the release process, which may decrease S2. Further experiments are needed to evaluate this process. These data are also complicated because lack of Ca^{2+} may affect Na^+-Ca^{2+} exchange mechanisms which may enhance non-Ca^{2+} dependent release.

Discussion

In the present experiments, we established the best stimulation paradigm for eliciting an easily detectable and reliable signal. In these experiments, GABA release from hippocampal slices was both frequency and time dependent. Significant stimulated GABA release occurred by exposing hippocampal slices to

10, 20 or 30 Hz electrical pulses for 2 min. (Fig 4-1) or 30 Hz electrical pulse for 1, 2 or 3 min (Fig 4-2). Among each stimulation condition, it appeared that 2 min of 30 Hz pulses and 3 min of 30 Hz pulses both elicited the largest and most reliable amounts of tritium release measured as large S1 and S2 values. Although 3 min of 30 Hz electrical pulse elicited the largest S1 and S2 individually, the ratio of S2/S1 is much smaller under this stimulation condition compared to 2 min of 30 Hz electrical pulse (Table 4-2). Therefore, considering S1, S2 and S2/S1 value, the 2 min of 30 Hz electrical pulse was considered ideal for our future studies.

Electrical stimulation has been frequently used in the past to elicit release of GABA from brain slices and synaptosomes. Electrical stimulation of brain slices is experimentally rather difficult to accomplish properly and there is so far no unanimous opinion on the characteristics of applied stimulation. Electrical stimulation increases several-fold the release of GABA from slices from different brain areas (Oja et al., 1977). The response to electrical stimulation is dependent on the range of frequency and stimulation time. It is important that the efflux of ^{3}H-GABA was increased by electrical stimulation, since this procedure is known to cause depolarization of neurons in brain slices (Hilman & Mcilwain, 1961). It is possible that the release mechanism for GABA is only stimulated by the rapid changes in cell polarity produced by rectangular pulses (Mcilwain et al., 1955).

We also tested whether electrically-stimulated ^{3}H-GABA release was calcium dependent by withdrawing calcium from the buffer. We found that spontaneous release of labeled GABA increased while stimulated GABA release was suppressed by about 50% in Ca^{++} - free solution. These data are consistent with similar findings that electrically-stimulated ^{3}H-GABA release was partially Ca^{2+}-dependent in rat cortical slices (Szerb et al., 1979) as well as from synaptosomal fractions and brain slices (Arias et al., 1984).

These results indicate that electrically-stimulated ^{3}H-GABA release from rat hippocampal slices in the presence of Ca^{2+} may come from both Ca^{2+}-dependent and Ca^{2+}-independent stores. The Ca^{2+}-dependent GABA release comes from vesicular stores. Ca^{2+} ions are considered a prerequisite for exocytotic emptying of storage granules of transmitters into synaptic clefts. When the cytoplasmic free Ca^{2+} concentration is elevated, a calcium channel is activated and triggers the GABA release from vesicular stores. Therefore, GABA release from vesicular stores occurs by an exocytotic mechanism that is Ca^{2+}-dependent. The Ca^{2+}-independent GABA release is thought to come from cytoplasmic GABA stores due to a Na^+-dependent mechanism which is independent of Ca^{2+} (Levi et al., 1978; Sandoval, 1980). When the cytoplasmic free Na^+ concentration is elevated, the Na^+-dependent GABA transporter operates in reverse and triggers the GABA release from cytoplasmic pools. Therefore, GABA release from these cytoplasmic pools is Na^+ dependent.

Removal of Ca^{2+} from the buffer also increased basal release. In hippocampal slices, spontaneous release of preloaded GABA has been shown to be increased by Ca^{2+} withdrawal (Peris et al., 1987; Minc-Golomb et al., 1988). The enhancement of spontaneous release is totally blocked by tetrodotoxin (Minc-

Golomb et al., 1988), therefore, it was suggested that the enhancement of spontaneous release was dependent on increased influx of Na^+ ions across destabilized plasma membranes (Saransaari & Oja, 1992). The significant change in basal release may complicate the interpretation of Ca^{2+} dependency of stimulated GABA release based on the reasons presented in the results. Further experiments are required to more fully test this hypothesis. Blockade of the Na^+-dependent uptake system during experiments to prevent its reversal may help clarify the Ca^{2+}-dependency of ^3H-GABA release. With the above cautions, the release of GABA appeared only partially Ca^{2+}-dependent.

On the other hand, it has been shown that endogenous GABA release is Ca^{2+}-dependent regardless of stimulation parameters. Endogenous GABA release from slices of guinea-pig cerebral cortex (Potashner, 1978), hippocampal CA1 subslices (Kampbuis et al., 1990), and cultured cerebellum neuron (Rogers et al., 1990) whether evoked by electrical or potassium stimulation, is generally 80-90% Ca^{++} -dependent. Perhaps, exogenous labelling by ^3H-GABA increases the stores of GABA present in cytoplasmic pools susceptible to Ca^{2+}-independent transporter-mediated release mechanisms.

These results suggest that electrically-stimulated ^3H-GABA release is partialy Ca^{2+} dependent. In the presence of Ca^{2+}, electrically-stimulated ^3H-GABA release came from Ca^{2+} dependent stores as well as Ca^{2+} independent stores. However, in the absence of Ca^{2+} electrically-stimulated ^3H-GABA release was only from Ca^{2+}-independent stores, probably as result of an increased influx of Na^+

(Hammerstard & Cultler, 1972). When we measure electrically-stimulated ^{3}H-GABA release in present of Ca^{2+}, we must be conscious that there is a Ca^{2+}-independent cytoplasmic GABA release which may be affected differently than Ca^{2+}-dependent release by cholinergic systems or CET. Therefore, our subsequent data must be carefully interpreted. For the best results, Ca^{2+}-independent cytoplasmic GABA release by reversal of the Na^+ - coupled reuptake carriers should be carefully controlled.

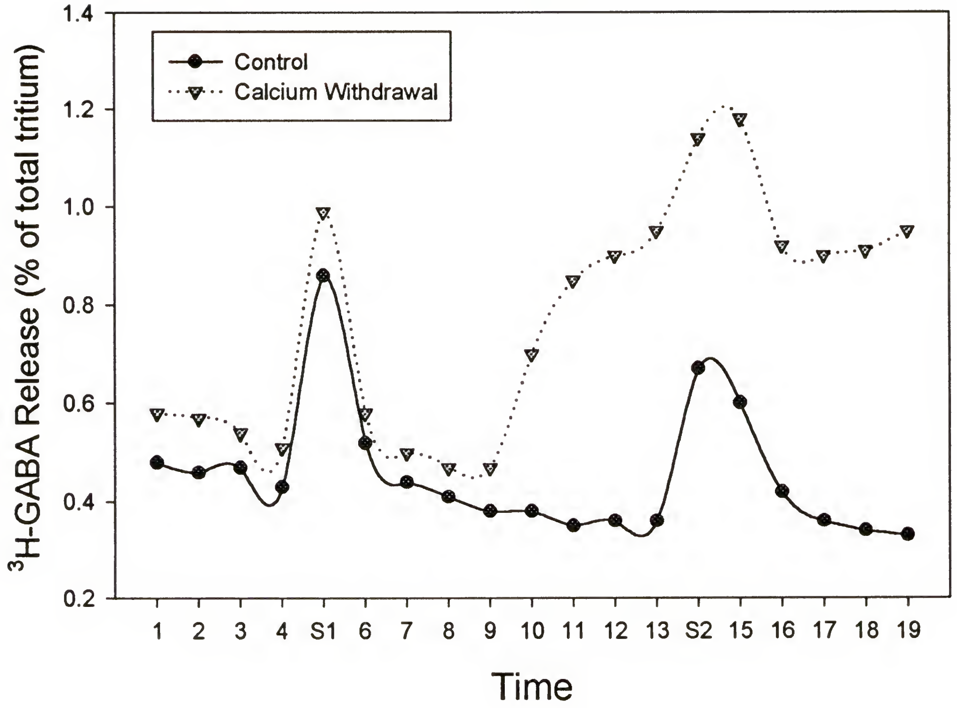

Figure 4-4. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence or absence of Ca^{++} in the buffer. The circles represent control and triangles represent Ca^{++} withdrawal. The Ca^{++} was withdrawn 20 min. before S2. The data are expressed as % of total tritium in slices at the time of collection. The values shown are means ± SEM with n=5. * $p < 0.05$ compared to each other.

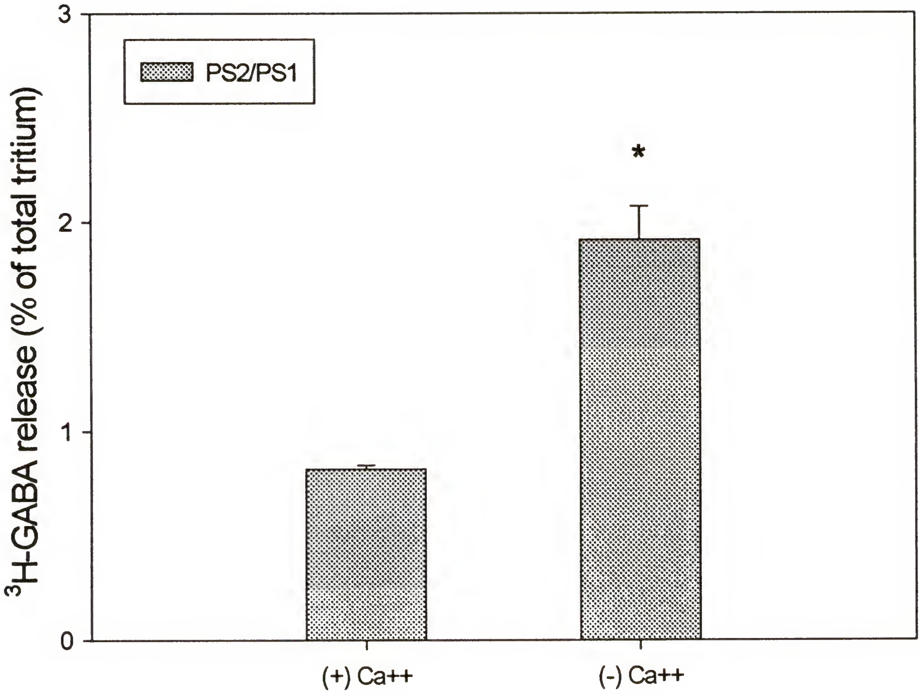

Figure 4-5. The basal ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence or absence of Ca^{++} in the buffer. The Ca^{++} was withdrawn 20 min. before S2. The data are expressed as PS2/PS1 ratio. The values shown are means ± SEM with n=5. * $p< 0.05$ compared to each other.

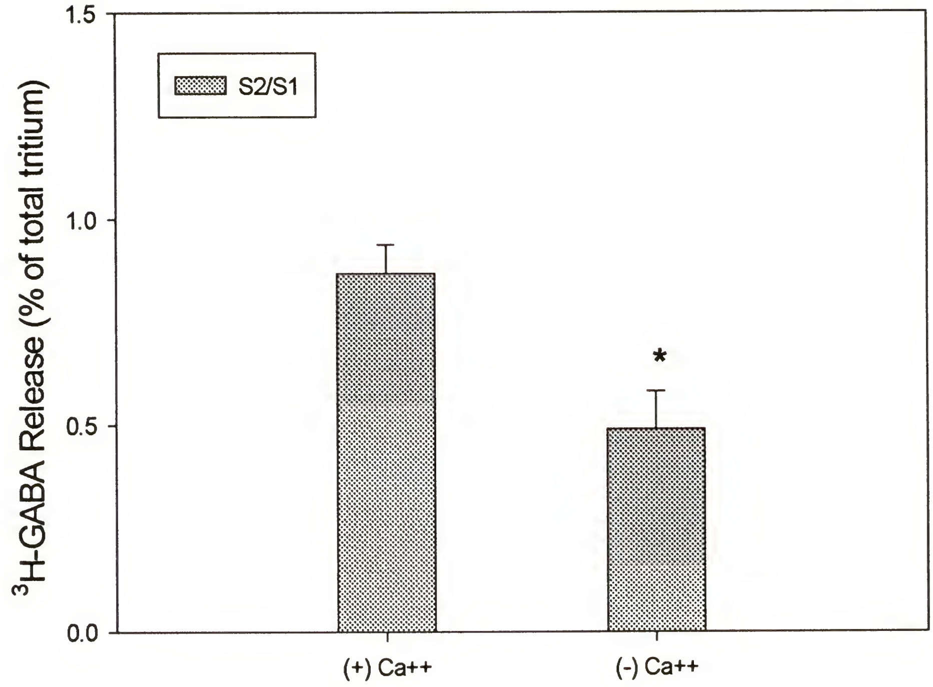

Figure 4-6. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence or absence of Ca^{++} in the buffer. The Ca^{++} was withdrawn 20 min. before S2. The data are expressed as S2/S1 ratio. The values shown are means ± SEM with n=5. * $p< 0.05$ compared to each other.

CHAPTER 5

THE EFFECT OF CHOLINERGIC RECEPTORS ON ELECTRICALLY-STIMULATED ^{3}H-GABA FROM HIPPOCAMPAL SLICES

Introduction

Cholinergic innervation of the hippocampus is widespread, underlying the multi-faceted effects of ACh as a modulator of hippocampal transmission. It also has been shown that there is a strong cholinergic influence on GABAergic and other neurons in hippocampus (Baisden et al., 1984; Ferraro et al., 1997; Leranth & Frotscher, 1987). GABAergic neurons in CA1, CA3 and dentate gyrus of hippocampus express muscarinic cholinergic receptors (Van der zee, 1993) and more specifically, GABAergic interneurons in hippocampus express M2 muscarinic ACh receptors (Hajos et al., 1998). It has been reported that muscarinic receptors modulate GABA release in different brain regions. Muscarinic receptors stimulate GABA release in substantia gelatinosa of the rat spinal dorsal horn (Baba et al., 1998). In rat striatal nerve terminals, muscarinic receptors inhibit the GABA release.

Additionally, much evidence suggests that CET may induce a permanent loss or reduction in cholinergic function in hippocampus (Arendt et al., 1989; Rothberg & Hunter, 1991). Thus it is possible that cholinergic receptors may play important roles in the regulation of GABA release in hippocampus, and this regulation can be affected by CET.

The primary goal of this study was to determine whether cholinergic receptors modulate GABA release in hippocampus. This was accomplished by measuring the effects of the cholinergic agents on electrically-stimulated ^{3}H-GABA release in hippocampus. In these experiments, the regulation of GABA release by carbachol, a cholinergic agonist, atropine, a muscarinic antagonist and mecamylamine, a nicotinic antagonist was tested.

Methods

Animals

Adult male Sprague-Dawley and Long-Evans hooded rats (225-250 g) were obtained from Charles River Breeding Laboratories. Animals were housed one per cage in a controlled environment with a 7:00 A.M. to 7:00 P.M. light cycle and temperature of 24 °C. These rats were randomly assigned to each experimental group.

Drug treatment

^{3}H-GABA release was measured using the method described in Chapter 3. Superfusion with medium containing different concentrations of receptor agonists and antagonists was started 20 min after S1 (45 min after the start of collection) and continued at a constant level until slices were removed from the chambers. Carbachol, atropine and mecamylamine, when tested alone, were added 20 min after S1. In experiments in which the interaction of two drugs was tested, receptor antagonists were present throughout superfusion and carbachol was added 20 min after S1. Each concentration was tested on at least duplicate slices; at least three control slices (never exposed to drug) were included in each experiment.

ANOVA was used for all parametric analyses as appropriate followed by Dunnett's multiple comparisons. The distribution of S2/S1 data was normal. All S2/S1 data were statistically analyzed using two way ANOVA tests. Data is represented as mean±SEM with N equal to number of rats.

Results

As found previously (Peris et al.,1997; Tremwel, Hunter & Peris, 1994), basal ^{3}H-GABA release from hippocampal slices was stable over the 90 min superfusion period regardless of treatments. This was indicated by the fact that the basal release measured before S2 (PS2) was about 80% of that measured before S1 (PS1) regardless of treatment (Table 5-1). The total tritium in slices was also not changed regardless of treatment (Table 5-2).

Effects of carbachol on electrically-stimulated ^{3}H-GABA release in hippocampus

Presynaptic cholinergic regulation of electrically-stimulated ^{3}H-GABA release in hippocampus of Sprague-Dawley rats was tested by treating superfused hippocampal slices with selective cholinergic agonists. Carbachol, a cholinergic agonist, increased ^{3}H-GABA release from hippocampus in a concentration-related fashion with a maximum increase of GABA release at 10 μM (Figure 5-1 and Table 5-3). One-factor ANOVA showed a significant main effect of carbachol concentration ($F(4,13) = 4.7$; $p < 0.05$).

In order to determine which subtype of cholinergic receptor mediated this effect of carbachol, we measured the effects of carbachol in the presence of atropine, a selective muscarinic receptor antagonist. In these experiments, the antagonist was present throughout superfusion while carbachol was included 20 minutes prior to S2. When slices were superfused with 10 µM atropine, before and during exposure to 30 µM carbachol, there was no longer a significant effect of carbachol to enhance GABA release (Figure 5-1). One-factor ANOVA showed no significant main effect of carbachol with atropine present (F(1,13) = 0.61).

Then, we measured the effects of carbachol in the presence of mecamylamine, a selective nicotinic receptor antagonist. In these experiments, the antagonist was present throughout superfusion while carbachol was included 20 minutes prior to S2. When slices were superfused with 10 µM mecamylamine, before and during exposure to 30 µM carbachol, mecamylamine did not block the effects of carbachol. There was still a significant effect of carbachol to enhance GABA release (Figure 5-1). One-factor ANOVA showed significant main effect of carbachol with mecamylamine present (F(1,13)= 4.3; $p < 0.05$).

Effects of atropine on electrically-stimulated ^{3}H-GABA release in hippocampus

Next, we tested the effect of atropine on electrically-stimulated ^{3}H-GABA release. In these experiments, the antagonist was included 20 minutes prior to S2. Atropine alone modulated ^{3}H-GABA release in a biphasic concentration-dependent manner (Figure 5-2 and Table 5-3). At the lowest concentration tested (1 µM), atropine significantly increased ^{3}H-GABA release, whereas at concentrations of 3,10, and 30 µM, atropine significantly decreased ^{3}H-GABA release. One-factor ANOVA showed a significant main effect of atropine concentration (F(8,67) =

7.649; $p < 0.001$).

Effects of mecamylamine on electrically-stimulated ^{3}H-GABA release in hippocampus

Next, we tested the effect of mecamylamine on electrically-stimulated ^{3}H-GABA release. In these experiments, the antagonist was included 20 minutes prior to S2. Mecamylamine alone at 1,3 or 10 µM, did not significantly modulate electrically-stimulated ^{3}H-GABA release in hippocampus (Figure 5-3 and Table 5-3). One-factor ANOVA showed no significant main effect of mecamylamine concentration on GABA release ($F(4,28) = 1.649$).

Cholinergic modulation of electrically-stimulated ^{3}H-GABA release in hippocampal slices from Long-Evans rats

The effects of carbachol and atropine on electrically-stimulated ^{3}H-GABA release were also tested in hippocampal slices from Long-Evans rats. We found that carbachol increased ^{3}H-GABA release in a concentration-related fashion (Figure 5-4). One-factor ANOVA showed a significant main effect of carbachol concentration ($F(3,17) = 10.84$; $p < 0.001$). Atropine modulated ^{3}H-GABA release in a biphasic concentration-dependent manner (Figure 5-4). At the lowest concentration tested (1 µM), atropine significantly increased ^{3}H-GABA release, whereas 10 µM atropine significantly decreased ^{3}H-GABA release. One-factor ANOVA showed a significant main effect of atropine concentration ($F(3,14) = 14.20$; $p < 0.001$). These results are consistent with the results from Sprague-Dawley rats.

Discussion

The present experiments examined the cholinergic regulation of electrically-stimulated ^{3}H-GABA release from adult rat hippocampal slices. The findings of the present study are that carbachol, a cholinergic agonist, increased GABA release from rat hippocampal slices. Our data show that the effects of carbachol can be blocked by 10 μM atropine, but not by 10 μM mecamylamine. Thus carbachol is acting at muscarinic receptors in hippocampus to facilitate GABA release. These data are consistent with evidence that GABAergic neurons in rat dorsal hippocampus express muscarinic ACh receptors (Walker et al., 1980). Additionally, carbachol causes excitation of hippocampal GABAergic nonpyramidal cells in a direct monosynaptic manner (Pitler & Alger, 1992) and stimulation of cholinergic axons increases GABAergic inhibitory activity in hippocampus (Alger, 1991).

Atropine alone modulates GABA release in a biphasic concentration-dependent manner in hippocampus. These data indicate that more than one subtype of muscarinic receptor may control GABA release in an opposing fashion. In brain regions other than the hippocampus, muscarinic receptors modulate GABA release in a different manner dependent on the brain region. Muscarinic receptors stimulate the GABA release in substantia gelatinosa of the rat spinal dorsal horn (Baba et al., 1998). In rat striatal nerve terminals, muscarinic receptors inhibit GABA release (Hajos et al., 1998; Sugita et al., 1991). It has been reported that GABAergic interneurons in CA1, CA3 and dentate gyrus of hippocampus express muscarinic cholinergic receptors (Van der zee, 1993); and more specifically, GABAergic interneurons in hippocampus express M2 muscarinic ACh receptors (Hajos et al., 1998). Thus it is possible that cholinergic receptors may play important roles in the regulation of GABA release in hippocampus. The biphasic concentration-dependent manner of effects of atropine in regulation of GABA release in hippocampus suggest that muscarinic receptors stimulate the release of GABA in a complicated manner. It is possible that different subtypes of muscarinic receptors may be involved in these different regions and these different subtypes of muscarinic receptors may regulate GABA release in opposite directions. Therefore, the biphasic effects of atropine may be due to its mixed effects on different subtypes of muscarinic receptors. Further characterization of the effect of each muscarinic receptor subtype on GABA release is necessary.

It has been shown that Long-Evans rats are more sensitive to ethanol than Sprague-Dawley rat. We use Long-Evans rats as our chronic alcohol treatment animal model. The effects of carbachol and atropine on electrically-stimulated ^{3}H-GABA release were also tested in hippocampal slices from Long-Evans rats. Carbachol increased ^{3}H-GABA release in a concentration-related fashion (Figure 5-4), and atropine modulated ^{3}H-GABA release in a biphasic concentration-dependent manner (Figure 5-4). These results are consistent with the results from Sprague-Dawley rats. Therefore, in hippocampal slices from both animal strains, muscarinic receptors modulate GABA release in hippocampus.

Neuronal nicotinic ACh receptors are involved with cognition and have been shown to be potential targets for ethanol effects. Carbachol is not selective for muscarinic or nicotinic ACh receptors, thus, the effect of carbachol to increase ^{3}H-GABA release may be mediated by nicotinic receptors. However, there is no evidence that nicotinic ACh receptors are present on GABAergic interneurons in the hippocampus. Additionally, our data showed that mecamylamine failed to block the effect of carbachol, and mecamylamine alone did not modulate ^{3}H-GABA release. These data indicate that nicotinic receptors are probably not involved in presynaptic regulation of GABA release in hippocampus although more definitive evidence should be obtained by testing the effects of specific nicotinic agonists. However, at this point, it appears that CET-induced increases in hippocampal GABA release probably do not involve changes in nicotinic ACh receptor regulation.

In conclusion, these data demonstrate that presynaptic muscarinic cholinergic receptors may play a facilitating role in the regulation of GABA release in the hippocampus while, nicotinic receptors do not exhibit robust regulation of GABA release.

Table 5-1. The basal ^{3}H-GABA release from hippocampal slices of adult rats was not affected by drug treatment. Basal release was measured before slices were exposed to each electrical field stimulation (PS1 and PS2). Drug (µM) was introduced prior to S2. The values shown are mean ± SEM of PS2/PS1 ratio (n=5-30 for each group).

PS2/PS1 Ratio

(µM)	Carbachol	Atropine	MMA
Control	0.75 ±0.05	0.77±0.05	0.79±0.04
0.1		0.77±0.01	
0.3		0.80±0.03	
1.0	0.79 ±0.01	0.77 ±0.01	0.81±0.03
3.0	0.78 ±0.03	0.80±0.01	0.81±0.03
10.0	0.81 ±0.03	0.77±0.02	0.79±0.01
30.0	0.80 ±0.03	0.75±0.01	
100.0		0.73±0.01	

Table 5-2. The total tissue ^{3}H-GABA in hippocampal slices of adult rats was not affected by drug treatment. After superfusion, the slices were solubilized, and total ^{3}H-GABA was determined by calculating the ^{3}H-GABA in both tissue and superfusate samples. The values shown are mean ± SEM of total ^{3}H-GABA (n=5-30 for each group).

Total ^{3}H-GABA

(µM)	Carbachol	Atropine	MMA
Control	131054±11756	120054±9756	114054±12775
0.1		111231±13056	
0.3		91054±11457	
1.0	110215±13432	101055±10845	99100±11277
3.0	123105±14523	141010±11677	141032±11451
10.0	143110±9117	105414±7568	102131±7569
30.0	139021±13725	121231±12543	
100.0		161124±15632	

Table 5-3. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the ACh agents, carbachol, atropine and mecamylamine. Drug was included 20 min prior to S2. There was a significant increase in stimulated GABA release in response to carbachol. Atropine modulated ^{3}H-GABA release in a biphasic dose dependent manner. Mecamylamine did not modulated ^{3}H-GABA release. Values shown are means ± SEM of S2/S1 ratio for N=5-30. * $P < 0.05$ compared to release in the absence of drugs.

S2/S1 Ratio

(μM)	Carbachol	Atropine	MMA
Control	0.75 ±0.05	0.77±0.04	0.67±0.02
0.1		0.82±0.09	
0.3		0.79±0.11	
1.0	0.79 ±0.06	1.06 ±0.11 *	0.73±0.08
3.0	0.96 ±0.07 *	0.64±0.06 *	0.67±0.06
10.0	0.98 ±0.05 *	0.58±0.05 *	0.78±0.05
30.0	0.95 ±0.04 *	0.49±0.05 *	
100.0		0.22±0.05 *	

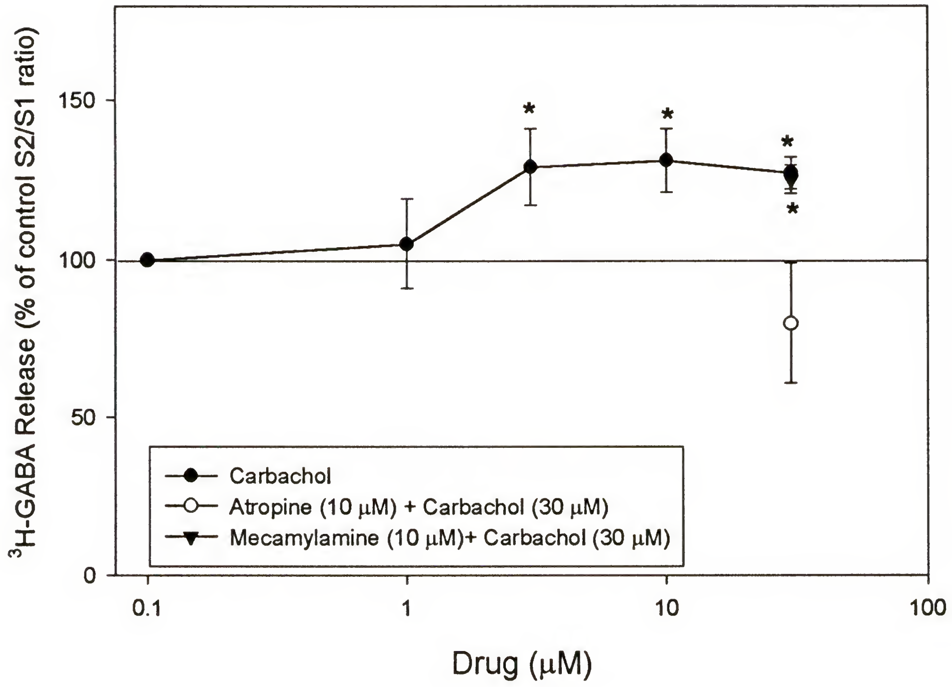

Figure 5-1. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the ACh agonist, carbachol. Carbachol was included 20 min prior to S2. There was a significant increase in stimulated GABA release in response to carbachol. Atropine (10 μM) blocked the effects of carbachol. Mecamylamine (10 μM) did not block the effects of carbachol. Concentrations of drug used were 0.1, 1, 3, 10 and 30 μM carbachol. Values shown are means ± SEM for N = 4-6. * $P < 0.05$ compared to release in the absence of drugs.

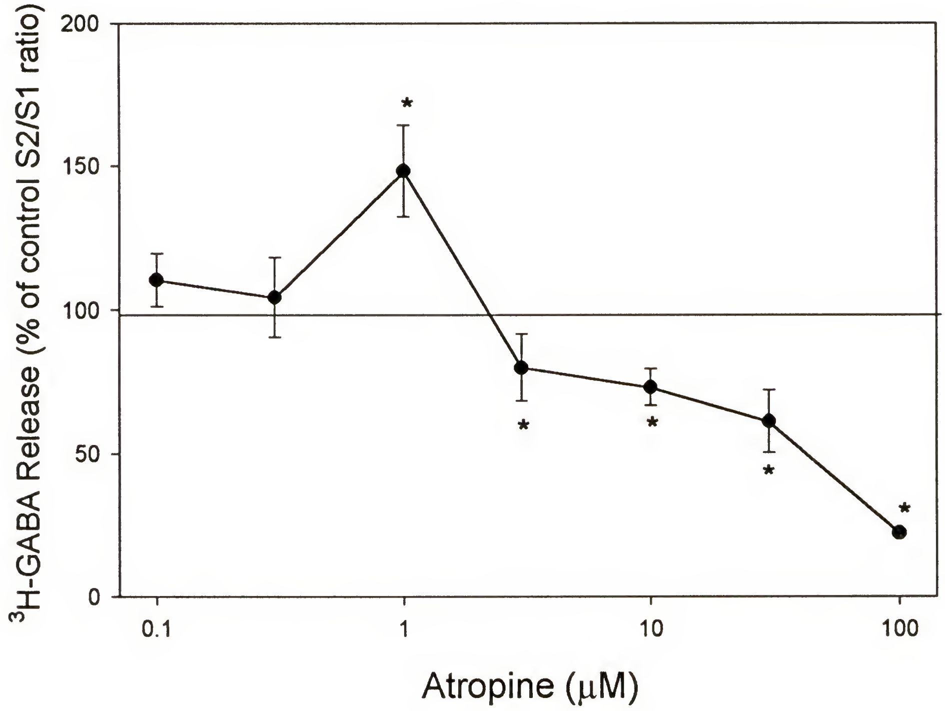

Figure 5-2. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the ACh antagonist, atropine. Atropine was included 20 min prior to S2. Atropine modulated ^{3}H-GABA release in a biphasic dose dependent manner. Concentrations of drug used were 0.1, 0.3,1, 3, 10, 30 and 100 μM for atropine. The values shown are means ± SEM for N=2-8. * $p < 0.05$ compared with control.

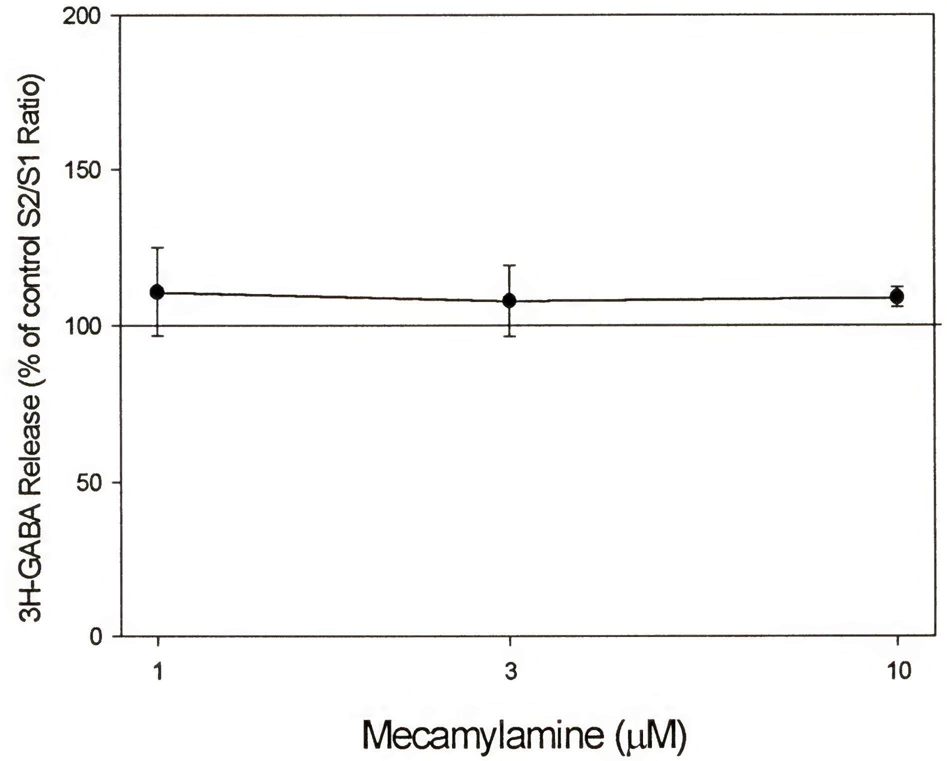

Figure 5-3. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the nicotinic ACh antagonist, mecamylamine. Mecamylamine did not modulate electrically-stimulated ^{3}H-GABA release from hippocampal slices. Concentrations of drug used were 1, 3, 10 and 30 μM mecamylamine. The values shown are means ± SEM for N=2-8. * $p< 0.05$ compared with control.

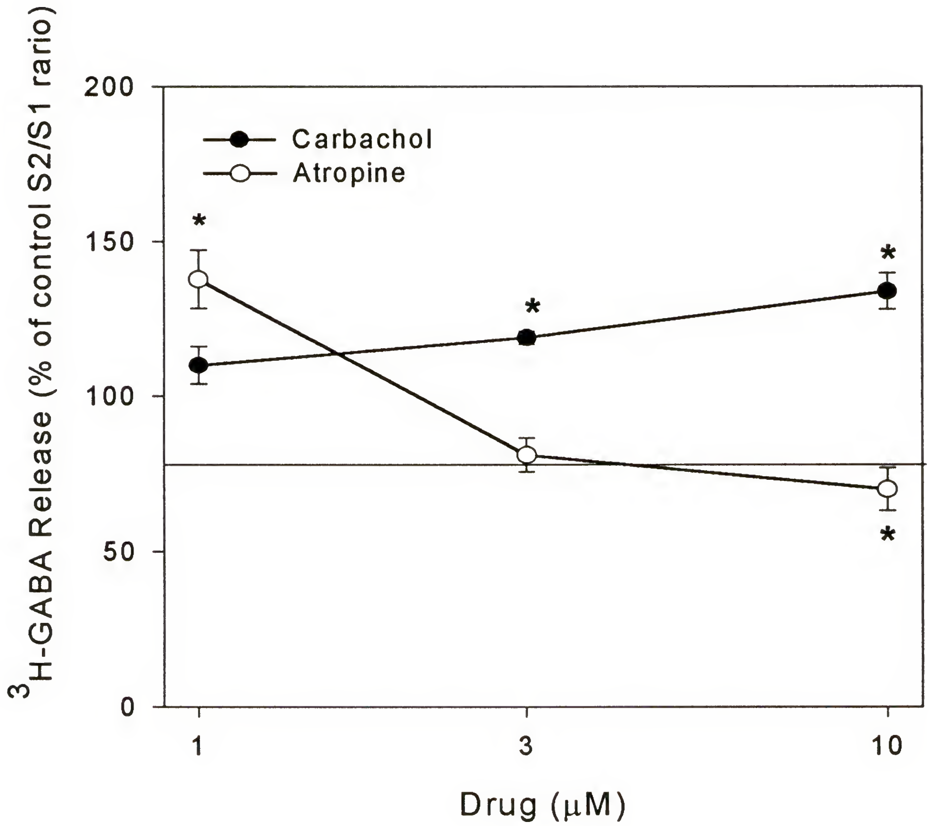

Figure 5-4. Electrically-stimulated ^{3}H-GABA release from hippocampus of Long-Evans rats was measured in the presence of the ACh agonist, carbachol and antagonist, atropine. Carbachol increased ^{3}H-GABA release in a dose dependent manner and atropine modulated ^{3}H-GABA release in a biphasic-dose dependent manner. Concentrations of drug used were 1, 3, 10 μM. The values shown are means ± SEM for N=5. * $p < 0.05$ compared with control.

CHAPTER 6

THE EFFECTS OF CET ON MUSCARINIC RECEPTOR REGULATION OF ^{3}H-GABA RELEASE FROM HIPPOCAMPAL SLICES OF SUCROSE- AND ETHANOL-TREATED RATS

Introduction

Cholinergic projections to the hippocampus from the basal forebrain appear to play a critical role in certain types of LTP. CET damages the septohippocampal projection resulting in loss of basal forebrain neurons, which is paralleled by a decline in ACh, choline acetyl transferase and acetylcholinesterase in the forebrain and hippocampus (Arendt et al., 1989). Losses of chemically specific afferents, including cholinergic afferents, often elicit postsynaptic changes which appear to at least partially compensate for the loss of input, i.e. receptor supersensitivity (Bird & Aghajanian, 1975). CET, however, results in structural as well as biochemical damage to surviving hippocampal neurons (Walker et al., 1993a,1993b). It has therefore been unclear whether the damage to the septohippocampal cholinergic projection produced by CET is accompanied by corresponding changes in cholinergic responses in surviving hippocampal neurons. Although Rothberg et al., (1992) showed that CET fails to alter the number of any specific muscarinic receptor subtype in hippocampus, chronic ethanol may act to alter processes downstream from the muscarinic receptor such as second messengers or ion channels.

In previous studies, we found that muscarinic receptors facilitate GABA release in hippocampus, but in a complicated manner. The primary goal of this study was to determine whether CET alters the sensitivity of muscarinic receptors to cholinergic agents that modulate GABA release in hippocampus. Our hypothesis is that this regulatory effect of muscarinic receptors in GABA release may be altered by CET. To test this hypothesis, we measured effects of carbachol and atropine on ^{3}H-GABA release in sucrose- and ethanol-treated rats followed by a 0- or 48-hour withdrawal period.

Methods

Animals

Long-Evans rats were treated with ethanol or sucrose diet as described in Chapter 3. After 28 weeks of treatment, blood ethanol levels ranged between 150-200 mg/dl measured at 8 A.M. The average body weight was 670±23 g for CET treated rats and 704±21 g for sucrose treated rats. Animals were treated with ethanol for 28 weeks followed by a 0 or 48 hour withdrawal period before decapitation as described in general methods. All animals survived after 48 hour withdrawal from 28 weeks ethanol treatment.

Drug treatment

^{3}H-GABA release were measured using a method described in Chapter 3. Superfusion with medium containing different concentrations of receptor agonists and antagonists was started 20 min after S1 (45 min after the start of collection) and continued at a constant level until slices were removed from the chambers.

Carbachol and atropine were added 20 min after S1. Each concentration was tested on at least duplicate slices; at least three control slices (never exposed to drug) were included in each experiment.

ANOVA was used for all parametric analyses as appropriate followed by Dunnett's multiple comparisons. The distribution of S2/S1 data was normal. All S2/S1 data were statistically analyzed using two way ANOVA tests. Data is represented as mean±SEM with N equal to number of rats.

Results

As found previously (Peris et al.,1997; Tremwel, Hunter and Peris, 1994), basal ^{3}H-GABA release from hippocampal slices was stable over the 90 min superfusion period regardless of CET or drug treatments. This was indicated by the fact that the basal release measured before S2 (PS2) was about 75% of that measured before S1 (PS1) regardless of CET or drug treatment (Table 6-1). The total tissue tritium was not changed regardless of CET or drug treatments (Table 6-2).

The effects of carbachol and atropine on ^{3}H-GABA release in hippocampal slices of sucrose- and ethanol- treated rats followed by a 48 hour withdrawal period

First, sucrose- and ethanol-treated animals were decapitated after a 48 hour withdrawal period. In sucrose treated control rats, there was a slight increase in ^{3}H-GABA release caused by carbachol (Figure 6-1). When the effect of carbachol was measured in CET rats, there was a statistically significant increase in GABA release caused by 3 μM, 10 μM and 30 μM carbachol (Figure 6-1 and Table 6-3).

ANOVA revealed a significant interaction between ethanol treatment and carbachol (F(3,49) = 3.3; $p < 0.05$) which was due to a significant carbachol effect in CET rats (F(3,23) = 15.7; $p < 0.001$) but not in sucrose rats (F(3,25) = 1.7 N.S.).

As previously seen in Figures 5-2 and 5-4, the effects of atropine to modulate ^{3}H-GABA release from hippocampal slices of sucrose treated rats were biphasic (Figure 6-2). In CET rats, the biphasic effect of atropine to modulate ^{3}H-GABA release was lessened such that there was no longer a significant difference in the S2/S1 ratios between atropine-treated slices and control slices (Figure 6-2 and Table 6-3). Two-way ANOVA found no significant interaction between CET and atropine (F (3,47)=0.74; N.S.). There was a significant main effect of atropine (F(3,47)=3.98; $p < 0.05$), but no main effect of CET. However, when the effect of atropine was analyzed separately in sucrose and CET rats, there was a significant effect of atropine in sucrose rats (F(3,22) = 14.3; $p < 0.01$) but not in CET rats (F(3,24) =0.96; $p > 0.05$). There was no effect of CET on basal tritium release compared to sucrose-treated rats (Table 6-1).

The effects of carbachol and atropine on ^{3}H-GABA release in hippocampal slices of sucrose- and ethanol- treated rats followed by no withdraw period

In these experiments, sucrose- and ethanol-treated animals were decapitated with no withdrawal period from the liquid diet. The basal ^{3}H-GABA release from hippocampal slices was stable over the 90 min superfusion period regardless of treatments. This was indicated by the fact that the basal release measured before S2 (PS2) was about 75% of that measured before S1 (PS1) regardless of treatment (Table 6-4).

In sucrose treated control rats, there was a slight increase in ^{3}H-GABA release caused by 10 µM carbachol, and a significant decrease in ^{3}H-GABA release caused by 10 µM atropine. When the effects of carbachol and atropine were measured in CET rats, there was a statistically significant increase in GABA release caused by 10 µM carbachol compared to control slices while atropine no longer significantly affected release (Figure 6-3 and Table 6-5). ANOVA revealed a significant interaction between ethanol treatment and carbachol ($F(2,32) = 4.36$; $p < 0.05$) which was due to a significant carbachol effect in CET rats ($F(2,17) = 10.82$; $p < 0.001$) but not in sucrose rats ($F(2,17) = 1.7$; N.S.). There is a significant atropine effect in sucrose rats ($F(2,14) = 27.36$; $p < 0.001$), but not in CET rats ($F(2,14) = 0.11$; N.S.).

Discussion

Our results show that enhancement of ^{3}H-GABA release by carbachol was increased in CET rats compared to control rats (Figure 6-1). Surprisingly, the effects of atropine were lessened in similarly-treated rats (Figure 6-2). Thus, unlike the effects of GABA$_B$ agents, in which both agonists and antagonists indicate a decrease in the number or efficacy of autoreceptors (Peris et al., 1997), muscarinic agonists indicate an increase in receptor efficacy while the antagonist indicates a decrease in presynaptic receptor modulation of GABA release. One possible mechanism is that CET may decrease ACh release presynaptically, which would increase the effect of an exogenously applied agonist but decrease the effect of an exogenous antagonist. These results are consistent with data that suggests CET damages the septohippocampal projection resulting in loss of basal forebrain neurons and a decline in cholinergic markers in the forebrain and hippocampus (Arendt et al., 1989).

In these experiments, our control animals were chronically given high concentrations of sucrose in the liquid diet (sucrose comprised 35-39 % of the total caloric intake). Although the sucrose-treated animal model has been widely accepted as a control model in ethanol research (Walker & Freund, 1971; Walker et al., 1980), there is still a controversy about whether chronic sucrose may change neuronal activity in the brain. It has been shown that a high sucrose diet increases sympathetic activity and blood pressure (Gradin et al., 1988; kotchen et al., 1991). Sucrose consumption decreases central dopamine systems and 5-HT2 / D2 receptor interactions (see Montgomery & Grottick, 1999). Therefore, it is possible that sucrose may also alter cholinergic function. Our data shown that, unlike in naive rats, there was no detectable effect of carbachol in regulation of GABA release in sucrose control animals. These results suggested that chronic sucrose may suppress cholinergic function in hippocampus. Therefore, it is necessary to further determine the role of sucrose in this animal model.

The animals in our studies were treated with ethanol for 28 weeks followed by either a 0 hour or 48 hour withdrawal period. CET had the same effect on cholinergic modulation of GABA release regardless of whether withdrawal occurred or not. These data indicate that the changes in cholinergic modulation of GABA release are due to neuronal adaption to the 28 weeks of ethanol treatment and are not withdrawal-induced.

ACh has been shown to increase the population spike amplitude in stratum pyramidale and inhibits EPSPs in stratum radiatum (Rothberg and Hunter, 1991). CET decreases the effects of ACh on population spike amplitude but has no effect on inhibition of EPSPs. The latter effect is thought to be due to the presence of presynaptic muscarinic cholinergic receptors on the terminals of the stratum radiatum afferents thereby reducing neurotransmitter release. The effect on the population spike is thought to be mediated by a pharmacologically distinct set of receptors. Either way, these data do not support our evidence that presynaptic cholinergic receptor function is upregulated in CET rats. Similarly, both cholinergic disinhibition and recurrent inhibition are decreased by CET (Pitler et al., 1992). Again, it is possible that different subtypes of muscarinic receptors are involved in these processes and these subtypes are affected differently by CET.

Table 6-1. The basal ^{3}H-GABA release in hippocampal slices of sucrose- and ethanol- treated rats by a 48 hour withdrawal period was not affected by drug treatment. Release was measured before slices were exposed to each electrical field stimulation (PS1 and PS2). Drug (μM) was introduced prior to S2. The values shown are mean ± SEM of PS2/PS1 ratio (n=5-30 for each group).

PS2/PS1Ratio

(μM)	Sucrose Carbachol	Sucrose Atropine	CET Carbachol	CET Atropine
Control	0.71 ±0.03	0.81 ±0.05	0.69 ±0.03	0.77 ±0.01
1.00		0.79 ±0.06		0.72 ±0.03
3.00	0.75 ±0.04	0.72 ±0.04	0.79 ±0.05	0.82 ±0.06
10.00	0.79 ±0.05	0.76 ±0.05	0.77 ±0.07	0.71 ±0.04
30.00	0.77 ±0.03		0.75 ±0.03	

Table 6-2. The total tissue ^{3}H-GABA from hippocampal slices was not changed regardless of CET or drug treatments. After superfusion, the slices were solubilized, and total tritium was determined by calculating the tritium in both tissue and superfusate samples. The values shown are mean ± SEM of total ^{3}H-GABA (n=5-30 for each group).

Total ^{3}H-GABA

(µM)	Sucrose Carbachol	Sucrose Atropine	CET Carbachol	CET Atropine
Control	103246±9865	134712±11024	115482±12754	126344±8467
1.00		924167±51219		92417±5219
3.00	124167±11219	151672±9212	101364±10310	139451±10947
10.00	99478±6478	146146±14921	141982±19657	104164±11488
30.00	121988±40108		124991±15121	

Table 6-3. Electrically-stimulated ^{3}H-GABA release from hippocampal slices of sucrose- and ethanol- treated rats after a 48 hour withdrawal period was measured in the presence of the ACh agonist, carbachol or atropine. Drug (µM) was introduced prior to S2. The values shown are mean ± SEM of S2/S1 ratio (n=5-30 for each group). * $P < 0.05$ compared to release in the absence of drugs.

S2/S1 Ratio

(µM)	Sucrose Carbachol	Sucrose Atropine	CET Carbachol	CET Atropine
Control	0.73±0.04	0.72±0.03	0.67 ±0.03	0.79±0.04
1.00		1.08±0.07 *		0.87±0.07
3.00	0.79±0.03	0.86±0.03 *	0.92±0.03 *	0.77±0.10
10.00	0.89±0.05	0.58±0.04 *	1.12±0.08 *	0.69±0.10
30.00	0.78±0.07		0.85±0.06 *	

Table 6-4. The basal ^{3}H-GABA release in hippocampal slices of sucrose- and ethanol- treated rats after no withdrawal period was not affected by drug treatment. Release was measured before slices were exposed to each electrical field stimulation (PS1 and PS2). Drug (μM) was introduced prior to S2. The values shown are mean ± SEM of PS2/PS1 ratio (n=5 for each group).

PS2/PS1 Ratio

	Control	Carbachol	Atropine
Sucrose	0.81 ±0.03	0.77 ±0.05	0.75 ±0.03
CET	0.76 ±0.07	0.69 ±0.03	0.79 ±0.04

Table 6-5. Electrically-stimulated ^{3}H-GABA release from hippocampal slices of sucrose- and ethanol- treated rats after no withdrawal period was measured in the presence of carbachol or atropine. Drug (µM) was introduced prior to S2. The values shown are mean ± SEM of S2/S1 ratio (n=5-30 for each group). * $P < 0.05$ compared to release in the absence of drugs.

S2/S1 Ratio

	Control	Carbachol	Atropine
Sucrose	0.84±0.04	0.90±0.04	0.56±0.03 *
CET	0.64±0.04	0.98±0.08 *	0.62±0.05

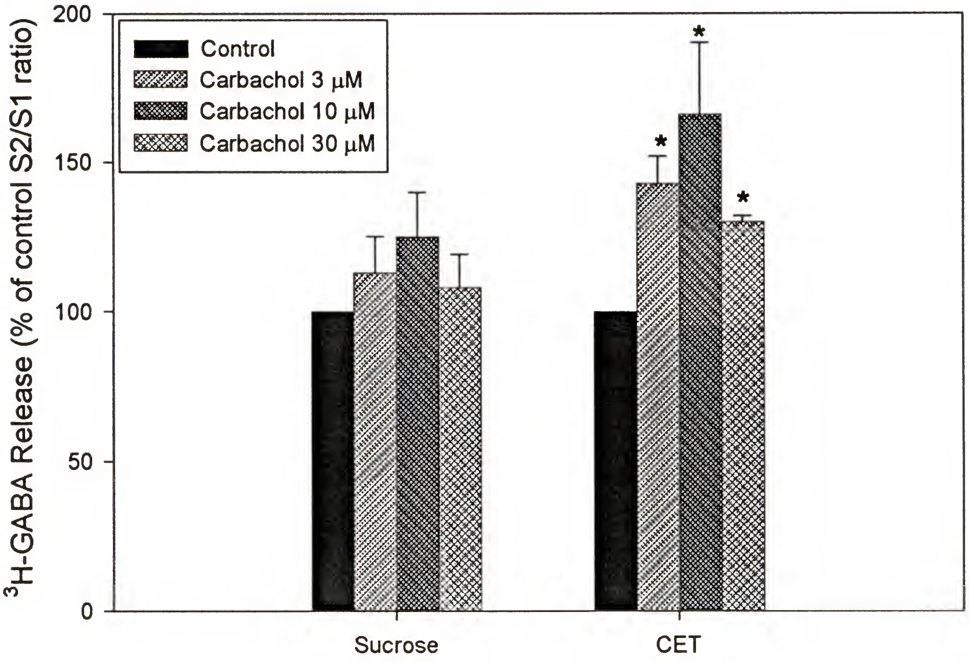

Figure 6-1. The effects of carbachol on ^{3}H-GABA release in sucrose- and ethanol-treated rats after a 48 hour withdrawal period from ethanol diet. ^{3}H-GABA release from hippocampus was measured as in Figure 5-1. The slices were exposed to 3.0, 10 and 30 μM carbachol or control buffer during S2. Carbachol significantly increased GABA release in CET rats but not in sucrose treated rats. The values shown are means + SEM for N = 5. * $P < 0.05$ compared to control values.

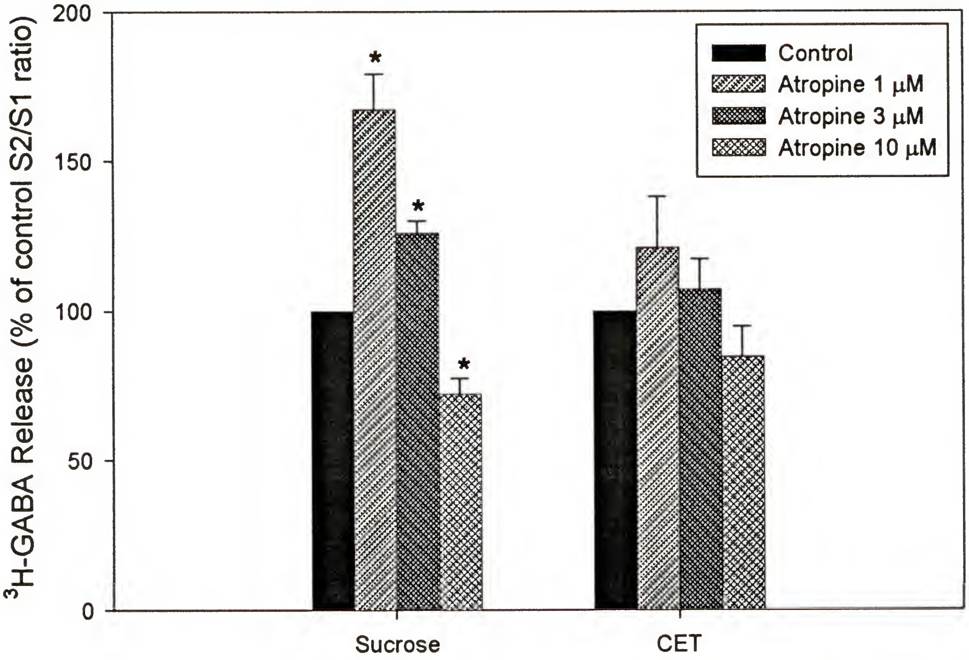

Figure 6-2. The effects of atropine on ^{3}H-GABA release in sucrose- and ethanol-treated rats after a 48 hour withdrawal period from ethanol diet. ^{3}H-GABA release from hippocampus was measured as in Figure 6-2. The slices were exposed to 1.0, 3.0, and 10 μM atropine or control buffer during S2. Atropine significantly_inhibited GABA release in sucrose treated rats but not CET rats . The values shown are means + SEM for N = 5. * $P < 0.05$ compared to control values.

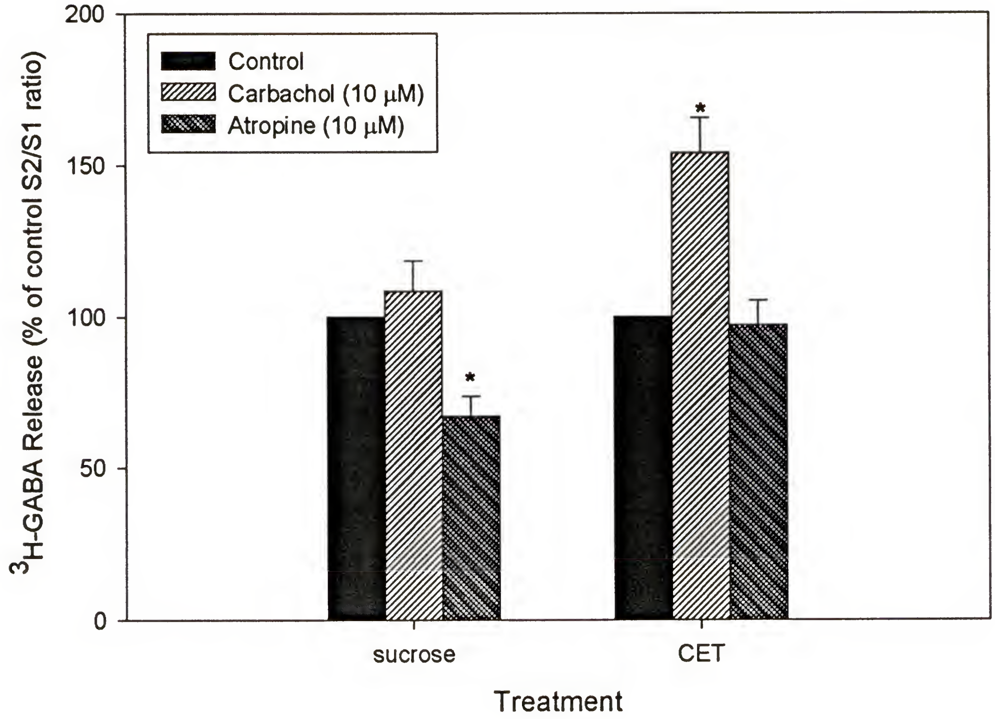

Figure 6-3. The effects of carbachol and atropine on ^{3}H-GABA release in sucrose- and ethanol-treated rats that were not withdrawn from the test diets. ^{3}H-GABA release from hippocampus was measured as in Figure 5-2. The slices were exposed to 10 μM carbachol, 10 μM atropine or control buffer during S2. The effect of carbachol was significantly enhanced by CET, whereas atropine no longer significantly inhibited GABA release in CET rats. The values shown are means ± SEM for N = 5. * $P < 0.05$ compared to control values.

CHAPTER 7
THE EFFECTS OF CET ON ELECTRICALLY-STIMULATED ^{3}H-ACH RELEASE AND CHOLINERGIC AUTORECEPTOR FUNCTION IN HIPPOCAMPAL SLICES.

Introduction

Previous studies show that enhancement of ^{3}H-GABA release by carbachol was significantly increased in slices from CET rats compared to sucrose fed control rats (Figure 6-1). The effects of atropine were lessened in similarly-treated rats (Figure 6-2). Thus, the effects of muscarinic agonists indicate an increase in receptor efficacy while the antagonist indicates a decrease in presynaptic receptor modulation of GABA release. These results suggest that CET may decrease ACh release presynaptically, which would increase the effect of an exogenously applied agonist but decrease the effect of an exogenous antagonist.

The primary goal of this study was to determine whether CET presynaptically alters ^{3}H-ACh release due to a loss of cholinergic input in hippocampus or alters the sensitivity of muscarinic autoreceptors and regulation of ^{3}H-ACh release. This was accomplished by measuring the effects of the cholinergic agents, carbachol and atropine on ^{3}H-ACh release in sucrose- and ethanol-treated rats followed by a 48 hour withdrawal period.

Methods

Animals

Long-Evans rats were treated with ethanol or sucrose diet as described in Chapter 2, general methods. After 28 weeks treatment, blood ethanol levels ranged between 150-200 mg/dl measured at 8 A.M. The average body weight was 770±34 g for CET treated rats and 801±31 g for sucrose treated rats. Animals were treated with ethanol for 28 weeks followed by a 48 hour withdrawal period before decapitation as described in methods. All animals survived after 48 hour withdrawal from 28 weeks ethanol treatment.

Drug treatment

^{3}H-ACh release were measured using an adaptation of a method described in Chapter 3. Briefly, these slices were incubated in Krebs' solution for 30 min. Then the medium was replaced with 4 ml fresh buffer containing 10 µl of ^{3}H-choline (85 Ci/mmol; final concentration about 30 nM). After 30 min incubation, 0.1 mM physostigmin (an Ach-esterase inhibitor) was added to the Krebs' buffer. Superfusion with medium containing different concentrations of receptor agonists and antagonists was started 20 min after S1 (45 min after the start of collection) and continued at a constant level until slices were removed from the chambers. Carbachol or atropine was added 20 min after S1. Each concentration was tested on at least duplicate slices; at least three control slices (never exposed to drug) were included in each experiment.

ANOVA was used for all parametric analyses as appropriate followed by

Dunnett's multiple comparisons. The distribution of S2/S1 data is normal. All S2/S1 data were statistically analyzed using two way ANOVA tests. Data is represented as mean±SEM with N equal to number of rats.

Results

Sucrose- and ethanol-treated animals were decapitated after a 48 hour withdrawal period. Basal and electrically-stimulated ^{3}H-ACh release from hippocampal slices were measured under conditions identical to those used for ^{3}H-GABA release. There was no effect of CET on basal (PS1) or electrically-stimulated (S1) ^{3}H-ACh release compared to sucrose rats (Figure 7-1). The total ^{3}H-ACh from hippocampal slices was not changed regardless of CET or drug treatments (Table 7-1).

Next, we measured the effects of carbachol or atropine on regulation of ^{3}H-ACh release from hippocampal slices of CET and sucrose rats. CET produced no detectable change in the functional operation of presynaptic muscarinic cholinergic autoreceptors which serve to regulate ACh release (Figure 7-2). When ACh release was measured from hippocampal slices under conditions exactly like those used to measure changes in regulation of GABA release, there was no detectable effects of atropine in regulation of ^{3}H-ACh release, while carbachol significantly decreased ^{3}H-ACh release from hippocampal slices. In addition, there was no difference in the effects of carbachol or atropine to affect ACh release in sucrose and CET treated rats (Figure 7-2).

Discussion

The present experiments examined the effect of CET on stimulated ACh release and presynaptic muscarinic cholinergic autoreceptors which serve to regulate ACh release. There was no detectable effect of CET on the presynaptic regulation of stimulated ACh release (Figure 7-1), when measured under identical stimulation conditions as GABA release. In addition, CET produced no detectable change in presynaptic muscarinic cholinergic autoreceptors which serve to regulate ACh release (Figure 7-2). Therefore, in view of these findings, it appears that the effect of CET is selective for muscarinic receptors located on GABA interneurons, but not those located on cholinergic neurons in hippocampus.

Our previous results, as well as the literature, suggested that CET may decrease ACh release presynaptically, which would increase the effect of an exogenously applied agonist but decrease the effect of an exogenous antagonist (Figure 6-1). There might be several possible reasons for failing to detect any effect of CET in regulation of ACh release in our experiments. First of all, we measured ACh release by loading ^{3}H-choline into slices and this administration of exogenous choline could have masked a deficit in ACh synthesis caused by CET. Secondly, we measured ACh release under identical stimulation conditions as GABA release and this frequency and duration of electrical pulses may not be the best stimulation conditions for maximum detection of CET-induced differences in ACh release. Thirdly, we measured ACh release from whole hippocampal slices, and these results may not detect a CET-induced change in ACh release from a small distinct population of synaptic clefts. Additionally, we only tested one concentration of carbachol and atropine for regulation of ACh release which would not indicate a change in the affinity of presynaptic muscarinic cholinergic autoreceptors which serve to regulate ACh release (Figure 7-2). Therefore, more detailed studies are needed for measuring the effect of CET on ACh release and ACh autoreceptors in regulation ACh release in both sucrose and CET rats.

There is evidence showing that CET does not alter the muscarinic receptor subtype densities in hippocampus as determined by either immunoprecipitation of M1-5 subtypes (Rothberg et al., 1993) or by maximal ^{3}H-QNB binding or carbachol displacement of specific ^{3}H-QNB binding (Rothberg et al., 1996). Our results are not disproved by these studies, but instead, our data indicate that only the small population of muscarinic receptors located on GABAergic neurons might be altered by CET. Thus CET may increase GABA release in hippocampus by both a reduction in presynaptic $GABA_B$ autoreceptors on GABAergic terminals in hippocampus (Peris et al., 1997) as well as a shift in the function of muscarinic receptors on these GABAergic terminals in hippocampus to favor agonist activation over antagonist inactivation. It is also possible that different subtypes of muscarinic receptors regulate GABA release in opposite directions and CET may selectively alter that regulation.

Table 7-1. The total tissue ^{3}H-ACh from hippocampal slices was not changed regardless of CET or drug treatments. After superfusion, the slices were solubilized, and total tritium was determined by calculating the tritium in both tissue and superfusate samples. The values shown are mean ± SEM of total ^{3}H-GABA (n=5 for each group).

Total ^{3}H-ACh

	Sucrose	CET
Control	109782 ± 8865	113634 ± 9846
Carbachol (30.00 μM)	112924 ± 9109	92417 ± 5919
Atropine (10.00 μM)	124167 ± 10945	104511 ± 10947

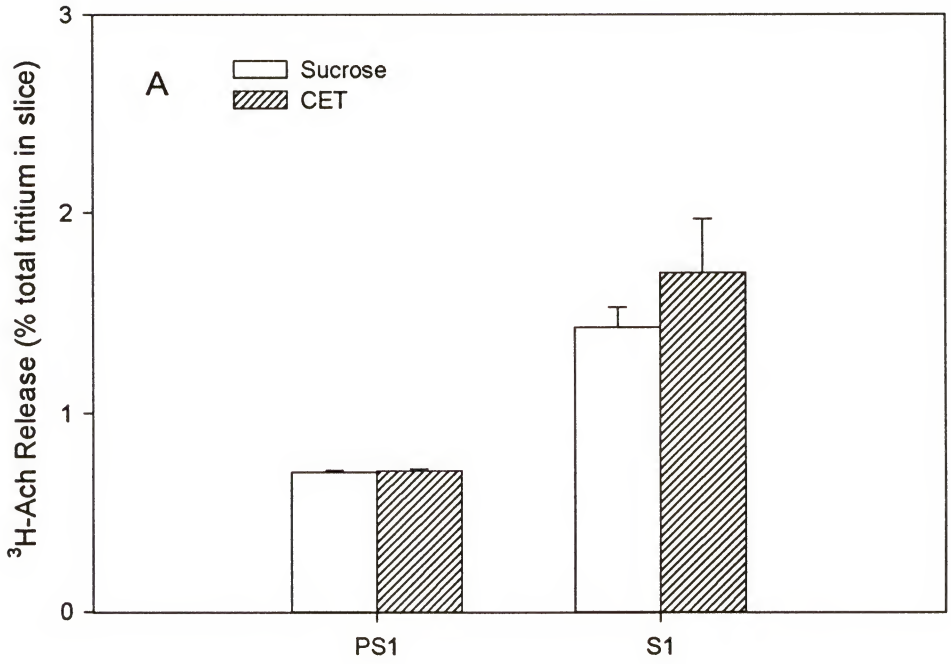

Figure 7-1. CET did not significantly change basal or electrically-stimulated ^{3}H-ACh release from superfused hippocampal slices. ^{3}H-ACh release from hippocampal slices was measured under conditions identical to those used in Figures 6-2 and 3. No significant differences were detected between CET and control rats. The values shown are means ± SEM for N = 5. * $p < 0.05$ compared to control values.

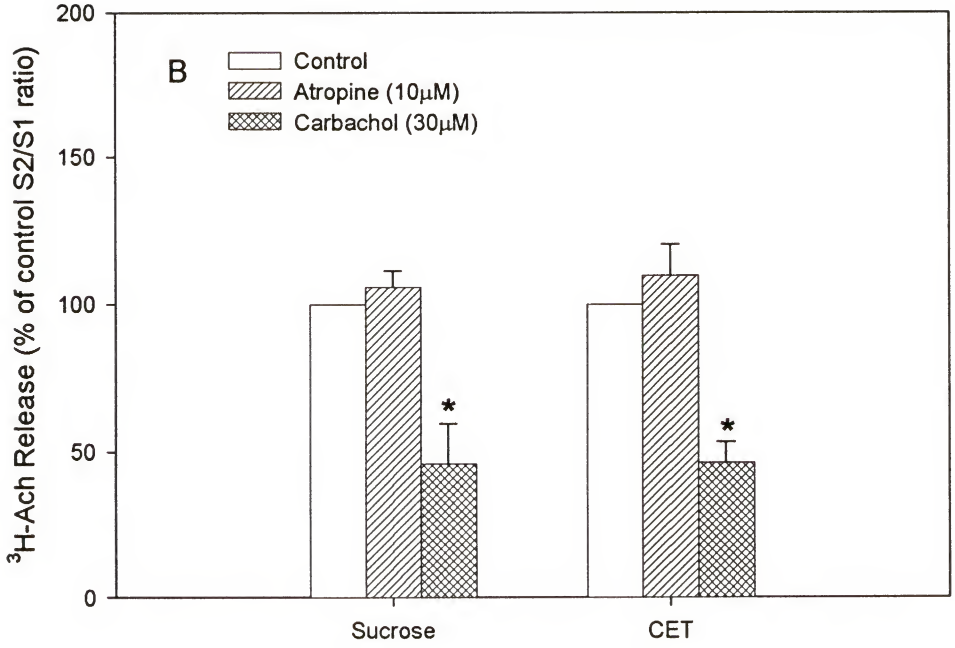

Figure 7-2. CET did not significantly change muscarinic autoreceptor modulation of electrically-stimulated ^{3}H-ACh release from superfused hippocampal slices. ^{3}H-ACh release from hippocampal slices was measured under conditions identical to those used in Figures 6-2 and 6-3. The slices were exposed to 30 μM carbachol, 10 μM atropine or control buffer during S2. No significant differences were detected between CET and control rats. The values shown are means ± SEM for N = 5. * $p < 0.05$ compared to control values.

CHAPTER 8

THE EFFECTS OF M1 and M2 MUSCARINIC RECEPTORS ON ELECTRICALLY-STIMULATED ^{3}H-GABA RELEASE IN HIPPOCAMPUS.

Introduction

There have been many reports of experiments using neurochemical techniques to show that muscarinic agonists and antagonists can modify the output of neurotransimitters (including ACh) from brain preparations. Nevertheless, there is still uncertainty about the types of muscarinic receptors involved in mediating these responses, mainly because most studies have not determined apparent antagonist dissociation constants. This is likely to lead to equivocal results, given the poor selectively of the available antagonists.

There has also been a controversy about the physiologic role of muscarinic subtypes in modulating GABA release from different brain regions. Again, this may be due to a lack of selective agonists and antagonists for a certain subtype of muscarinic receptor. It has been reported that M1 muscarinic receptors are involved in modulation of GABA release in different brain regions. M1 muscarinic receptors mediate inhibition of GABA release in rat cerebral cortex (Hashimoto et al., 1994), but facilitate GABA release in neostriatum (Harsing & Zigmond 1998). Using electrophysiological studies, Sugita et al. (1991) found that inhibition of GABA release involves an M1 receptor. M2 muscarinic receptors have been reported to mediate stimulation of GABA release in the rat substatia nigra (Kayadjanian et al., 1994).

Previous chapters have described that carbachol increases electrically-stimulated ^{3}H-GABA release in a concentration-dependent manner. Atropine at higher concentration (3-30 µM) decreased ^{3}H-GABA release while 1 µM atropine increase ^{3}H-GABA release. These results indicate that muscarinic receptors modulate GABA release in a complicated manner. Perhaps, different subtypes of muscarinic receptors are involved in regulation of GABA release.

Therefore, further studies of the regulation of GABA release by the different subtypes of muscarinic receptors in hippocampus will help us understand the mechanism of how CET alters the muscarinic regulation of GABA release. Furthermore, these data may help us to understand the mechanism underlying the disruption of LTP by CET. In these studies, we measured electrically-stimulated ^{3}H-GABA release from preloaded superfused hippocampal slices of naive rats with selective agonists and antagonists for muscarinic receptor subtypes.

Methods

Animals

Adult male Sprague-Dawley rats (225-250 g) were obtained from Charles River Breeding Laboratories. Animals were housed one per cage in a controlled environment with a 7:00 A.M. to 7:00 P.M. light cycle and temperature of 24°C. These rats were randomly assigned to each drug treatment group.

Drug Treatment

^{3}H-GABA release were measured using an adaptation of a method described in Chapter 3. Superfusion with medium containing different concentrations of receptor agonists and antagonists was started 20 min after S1 (45 min after the start of collection) and continued at a constant level until slices were removed from the chambers. McNA-363, a selective M1 muscarinic receptor agonist; pirenzipine, a selective M1 muscarinic receptor antagonist; oxotremorine, at a lower concentration (nM), a selective M2 muscarinic receptor agonist; and AF-DX116, a selective M2 muscarinic receptor antagonist, were each tested alone, added 20 min after S1. In some experiments, receptor antagonists were present throughout superfusion and agonist was added 20 min after S1. Each concentration was tested on at least duplicate slices; at least three control slices (never exposed to drug) were included in each experiment.

ANOVA was used for all parametric analyses as appropriate followed by Dunnett's multiple comparisons. The distribution of S2/S1 data is normal. All S2/S1 data were statistically analyzed using two way ANOVA tests. Data is represented as mean±SEM with N equal to number of rats.

Results

As seen in all previous experiments, basal ^{3}H-GABA release from hippocampal slices was stable over the 90 min superfusion period regardless of treatments. This was indicated by the fact that the basal release measured before S2 (PS2) was about 75% of that measured before S1 (PS1) regardless of treatment

(Table 8-1). The total tritium in slices was not changed regardless of drug treatments (Table 8-2).

Effects of McN-A-343 on Regulation of Electrically-Stimulated ^{3}H-GABA Release.

To characterize the specific role of subtypes of muscarinic receptors on regulation of GABA release from hippocampal slices of Sprague-Dawley rats, we tested the effect of McN-A-343, a selective M1 muscarinic receptor agonist, on regulation of ^{3}H-GABA release. McN-A-343 inhibited ^{3}H-GABA release from hippocampal slices in a concentration-related fashion with a maximum inhibition of GABA release at 1 μM (Figure 8-1 and Table 8-3). One-factor ANOVA showed a significant main effect of McN-A-343 concentration ($F(5,18) = 4.7$; $p < 0.05$). In order to determine which subtype of muscarinic receptor mediated this effect of McN-A-343, we measured the effects of McN-A-343 in the presence of pirenzipine, a selective M1 muscarinic receptor antagonist. In these experiments, the antagonist was present throughout superfusion while McN-A-343 was included 20 minutes prior to S2. When slices were superfused with 1 μM pirenzipine, before and during exposure to 1 μM McN-A-343, there was no longer a significant effect of McN-A-343 to enhance GABA release (Figure 8-1). One-factor ANOVA showed no significant main effect of McN-A-343 with pirenzipine present ($F(1,10) = 0.61$).

Next, we measured the effects of McN-A-343 in the presence of AF-DX 116, a selective M2 muscarinic receptor antagonist. In these experiments, the antagonist was present throughout superfusion while McN-A-343 was included 20 minutes prior to S2. When slices were superfused with 10 μM AF-DX 116, before and during exposure to 1 μM McN-A-343. 10 μM AF-DX 116 did not block the effects of 1 μM McN-A-343. There was still a significant effect of McN-A-343 to inhibit GABA release (Figure 8-1). One-factor ANOVA showed significant main effect of McN-A-343 with 10 μM AF-DX 116 present ($F(1,13)= 4.3$; $p < 0.05$).

Effects of Pirenzipine on Regulation of Electrically-Stimulated ^{3}H-GABA Release

Next, we tested the effect of pirenzipine on electrically-stimulated ^{3}H-GABA release. In these experiments, pirenzipine was included 20 minutes prior to S2. Pirenzipine alone increased ^{3}H-GABA release in a concentration-related fashion with a maximum increase of GABA release at 1 μM (Figure 8-2 and Table 8-3). One-factor ANOVA showed a significant main effect of pirenzipine concentration ($F(6,42) = 7.649$; $p < 0.001$). However, at the higher concentration tested (10 μM), the effect of pirenzipine no longer significantly modulated ^{3}H-GABA release.

Effects of Oxotremorine on Regulation of Electrically-Stimulated ^{3}H-GABA Release

In this study, we tested the effect of oxotremorine, a selective M2 muscarinic receptor agonist, on modulation of electrically-stimulated ^{3}H-GABA release in hippocampus of Sprague-Dawley rats. Oxotremorine was included 20 minutes prior to S2. Oxotremorine significantly increased ^{3}H-GABA release at lower concentrations but not at higher concentrations (Figure 8-3 and Table 8-3). At a concentration of 0.01 μM, oxotremorine maximally increased GABA release. One-factor ANOVA showed a significant main effect of oxotremorine concentration ($F(5,26) = 7.951$; $p < 0.01$).

In order to determine which subtype of muscarinic receptor mediated this effect of oxotremorine, we measured the effects of oxotremorine in the presence of AF-DX 116, a selective M2 muscarinic receptor antagonist. In these experiments, the antagonist was present throughout superfusion while oxotremorine was included 20 minutes prior to S2. When slices were superfused with 10 μM AF-DX 116, before and during exposure to 0.01 μM oxotremorine, there was no longer a significant effect of oxotremorine to enhance GABA release (Figure 8-3). One-factor ANOVA showed no significant main effect of oxotremorine with AF-DX 116 present ($F(1,10) = 0.61$).

We then measured the effects of oxotremorine in the presence of pirenzipine, a selective M1 muscarinic receptor antagonist. When slices were superfused with 1 μM pirenzipine, before and during exposure to 0.01 μM oxotremorine, pirenzipine did not block the effects of oxotremorine. There was still a significant effect of oxotremorine to enhance GABA release (Figure 8-3). One-factor ANOVA showed a significant main effect of oxotremorine with 1 μM pirenzipine present ($F(1,13)= 4.3$; $p < 0.05$).

Effects of AF-DX 116 Regulation of Electrically-Stimulated ^{3}H-GABA Release

Next, we tested the effect of AF-DX116 on electrically-stimulated ^{3}H-GABA release. AF-DX116 inhibited ^{3}H-GABA release in a concentration-related fashion (Figure 8-4 and Table 8-3). One-factor ANOVA showed a significant main effect of AF-DX116 concentration ($F(5,30) = 7.649$; $p < 0.001$). There was no effect of any of the above tested drugs on basal release at any concentration (Table 8-1).

Discussion

The present experiments examined the muscarinic receptor subtypes involved in the regulation of electrically-stimulated ^{3}H-GABA release from adult rat hippocampal slices. The findings of the present study are that McNA-343, a selective M1 muscarinic receptor agonist, significantly deceased ^{3}H-GABA release while pirenzipine, a selective M1 muscarinic receptor antagonist, significantly increased ^{3}H-GABA release. The effects of McNA-343 were blocked by an M1 muscarinic receptor selective antagonist, but not by an M2 muscarinic receptor selective antagonist. These results suggest that M1 muscarinic receptors may play an inhibiting role in regulation of GABA release in hippocampus. On the other hand, oxotremorine, at lower concentrations (nM), a selective M2 muscarinic receptor agonist, significantly increased ^{3}H-GABA release while AF-DX116 significantly decreased ^{3}H-GABA release in a dose-dependent manner. The effects of oxotremorine were blocked by the M2 muscarinic receptor selective antagonist, but not by the M1 muscarinic receptor selective antagonist. These results suggest that M2 muscarinic receptors may play a facilitory role in regulation of GABA release in hippocampus. The balance between these two receptors subtype may determine the activity of the GABA interneuron in nature.

Some studies have showed that M1 muscarinic receptors may mediate inhibition or facilitation of GABA release depending on brain regions. In rat cerebral cortex (Hashimoto et al., 1994), M1 muscarinic receptors mediated inhibition of GABA release, while in neostriatum they facilitated GABA release (Harsing and

Zigmond 1998). The effects of M2 muscarinic receptor regulation of GABA release are also regionally dependent. Kayadjanian et al., (1994) reported that M2 muscarinic receptor mediate stimulation of GABA release in the rat substantia nigra.

The previous chapter described that CET alters functional muscarinic receptor regulation of GABA release in hippocampus, but in a complex manner. This may be due to the fact that CET selectively alters the function of different subtypes of muscarinic receptors. Therefore, it is necessary to test how CET alters the effects of these muscarinic receptor subtypes.

Table 8-1. The basal ^{3}H-GABA release in hippocampal slices of adult rats was not affected by drug treatment. Release was measured before slices were exposed to each electrical field stimulation (PS1 and PS2). Drug (µM) was introduced prior to S2. The values shown are mean ± SEM of PS2/PS1 ratio (n=5-32 for each group).

PS2/PS1 Ratio

(µM)	AF-DX116	McN-A-343	Pirenzipine	Oxotremorine
Control	0.71±0.05	0.74±0.02	0.79±0.07	0.79±0.04
0.0003				0.77±0.05
0.001				0.82±0.08
0.003				0.71±0.04
0.01				0.75±0.05
0.03				0.70±0.06
0.10		0.72±0.05	0.77±0.05	0.75±0.05
0.30	0.74±0.04	0.75±0.03	0.75±0.03	0.81±0.09
1.0	0.76±0.02	0.81±0.06	0.74±0.04	
3.0	0.82±0.07	0.78±0.04	0.84±0.05	
10.0	0.69±0.02		0.71±0.06	
30.0	0.75±0.05			

Table 8-2. The total tissue ^{3}H-GABA from hippocampal slices of adult rats was not affected by drug treatment. After superfusion, the slices were solubilized, and total tritium was determined by calculating the tritium in both tissue and superfusate samples. The values shown are mean ± SEM of total ^{3}H-GABA (n=5-32 for each group).

Total ^{3}H-GABA

(µM)	AF-DX116	McN-A-343	Pirenzipine	Oxotremorine
Control	102469±11202	118874±9872	123579±10147	97979± 6594
0.0003				102357±9014
0.001				130082± 10148
0.003				134571±12304
0.01				167975±18145
0.03				131270±12346
0.10		132172±9987	132177±10120	98775±5423
0.30	120174±10914	99675±8749	102375±9812	106581±11059
1.0	79576±6542	1458681±9714	17174±13451	
3.0	129682±15747	114778±12694	109184±74521	
10.0	109869±9412		145971±16145	
30.0	136875±14095			

Table 8-3. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the selective muscarinic subtype agonists and antagonists, McN-A-343, pirenzipine, oxotremorine and AF-DX116. Drug was included 20 min prior to S2. Values shown are means ± SEM of S2/S1 ratio for N=5-30. * $P < 0.05$ compared to release in the absence of drugs.

S2/S1 Ratio

(µM)	AF-DX116	McN-A-343	Pirenzipine	Oxotremorine
Control	0.83±0.04	0.84±0.03	0.77±0.03	0.70±0.05
0.0003				0.77±0.05
0.001				0.82±0.08
0.003				0.82±0.07
0.01				1.42±0.10 *
0.03				1.18±0.18 *
0.10		0.81±0.05	0.78±0.07	088±0.15
0.30	0.74±0.04	0.62±0.02 *	0.91±0.06 *	0.81±0.09
1.0	0.69±0.05	0.54±0.03 *	1.09±0.13 *	
3.0	0.68±0.05 *	0.78±0.04	1.07±0.12 *	
10.0	0.54±0.03 *		0.75±0.04	
30.0	0.38±0.01 *			

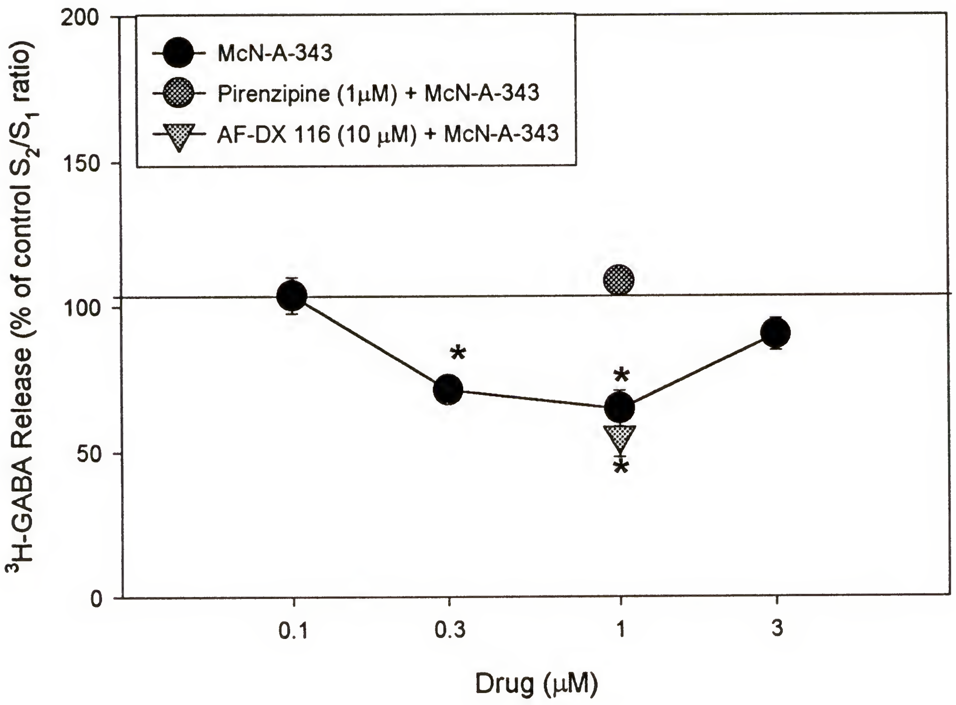

Figure 8-1. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the M1 muscarinic receptor agonist, McN-A-343. McN-A-343 was included 20 min prior to S2. There was a significant decrease in stimulated GABA release in response to McN-A-343. Pirenzipine (1 μM) blocked the effects of McN-A-343. AF-DX 116 (10 μM) did not block the effects of McN-A-343. Concentrations of McN-A-343 used were 0.1, 0.3, 1 and 3 μM. Values shown are means ± SEM for N = 4-6. * $P < 0.05$ compared to release in the absence of drugs.

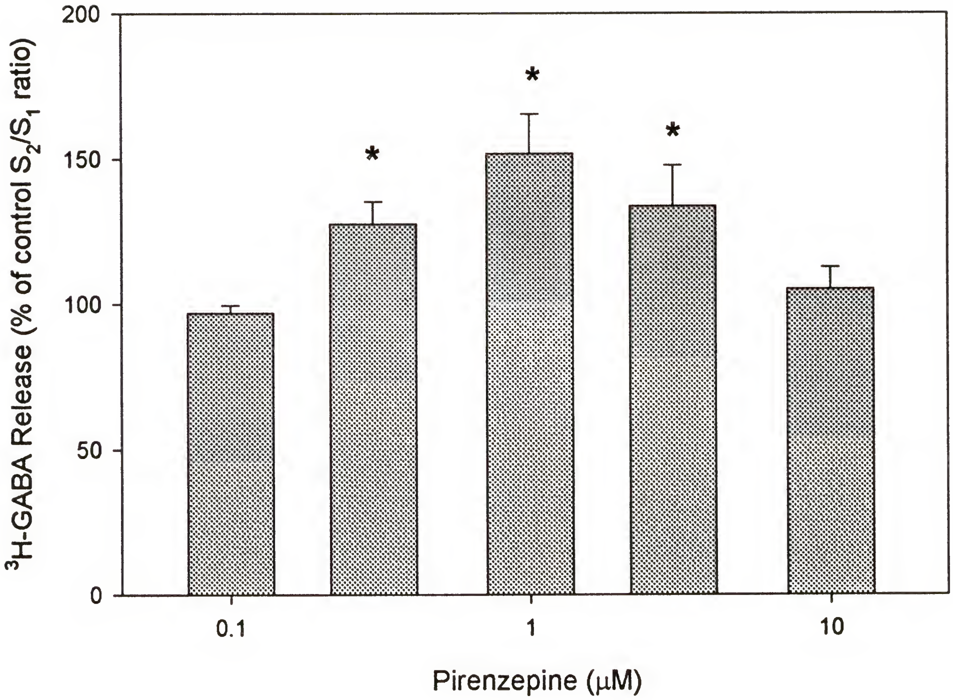

Figure 8-2. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the M1 muscarinic receptor antagonist, pirenzipine. There was a significant increase in stimulated GABA release in response to pirenzipine. Concentrations of drug used were 0.1, 0.3,1, 3 and 10 µM. The values shown are means ± SEM for N=4-8. * $p < 0.05$ compared with control.

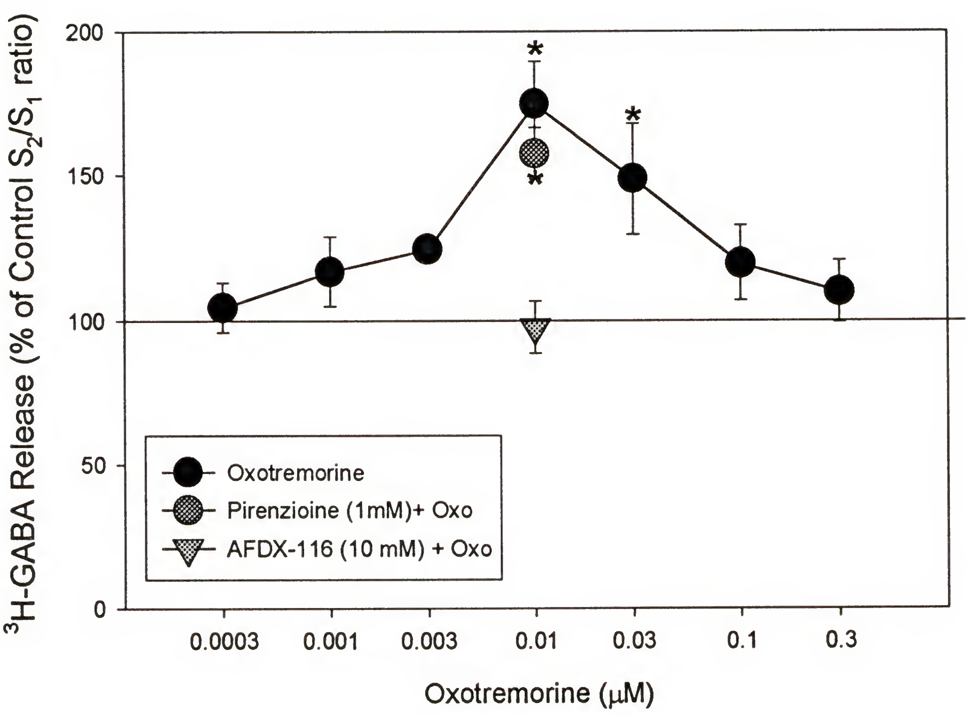

Figure 8-3. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the M2 muscarinic agonist, oxotremorine. Oxotremorine was included 20 min prior to S2. Oxotremorine significantly increased GABA release at 10 nM and 30 nM concentrations. The values shown are means ± SEM for 4-8. * $p < 0.05$ compared with control.

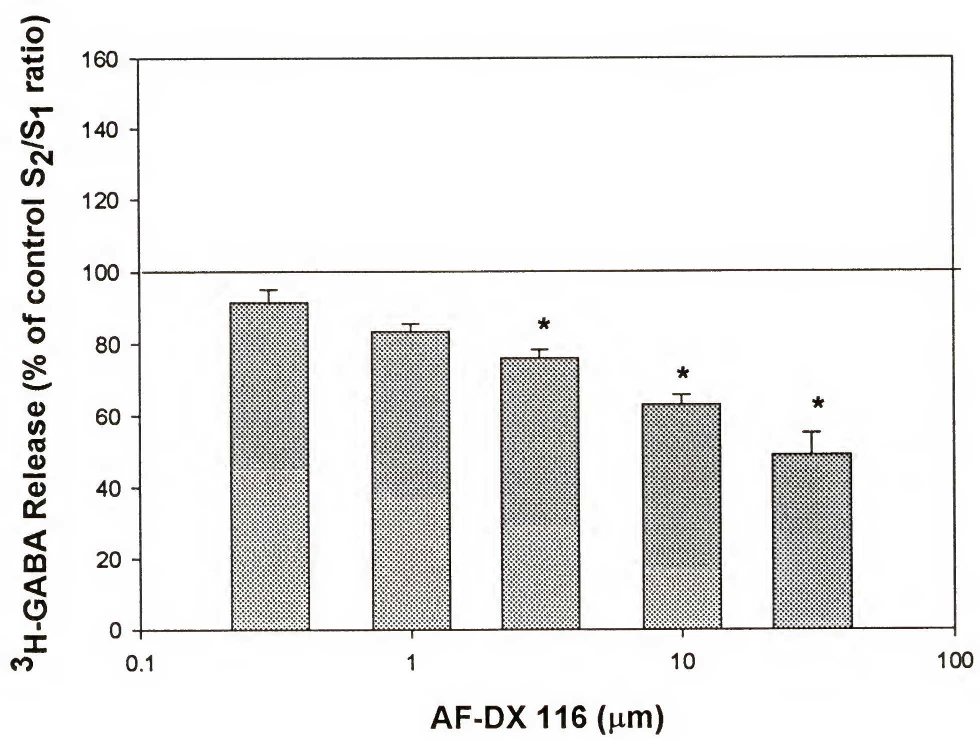

Figure 8-4. Electrically-stimulated ^{3}H-GABA release from hippocampus of Sprague-Dawley rats was measured in the presence of the M1 muscarinic receptor antagonist, AF-DX116. AF-DX116 inhibited the ^{3}H-GABA release in a dose dependent manner. Concentrations of drug used were 0.3,1, 3, 10 and 30 μM. The values shown are means ± SEM for N=3-8. * $p < 0.05$ compared with control.

CHAPTER 9

THE EFFECTS OF CET ON M1 AND M2 MUSCARINIC RECEPTOR REGULATION OF ^{3}H-GABA RELEASE IN HIPPOCAMPUS

Introduction

CET usually leads to regionally selective decreases in muscarinic receptor density (see Shanley & Wilce, 1993). CET decreases the effects of ACh on population spike amplitude but has no effects on carbachol-inhibition of EPSPs (Rothberg & Hunter, 1991). Additionally, both cholinergic disinhibition and recurrent inhibition are decreased by CET. These data imply that CET may decrease the number or functional status of muscarinic cholinergic receptors in hippocampus. Evidence indicates this is not due to an effect of CET on muscarinic subtype densities (Rothberg et al., 1993; Rothberg et al.,1996). The effect of carbachol on the EPSP is thought to be due to the presence of presynaptic muscarinic cholinergic receptors on the terminals of the stratum radiatum afferents. When these receptors are activated, neurotransmitter release is reduced. Thus, the effects of CET seem to be selective for postsynaptic rather than presynaptic cholinergic receptors.

Previous studies show that enhancement of ^{3}H-GABA release by carbachol was significantly increased in slices from CET rats compared to sucrose fed control rats (Figure 6-1 and Figure 6-3). The effects of atropine were lessened in similarly-treated rats (Figure 6-2 and Figure 6-3). Therefore, regulation of GABA release from CET rats by muscarinic agonists indicates an up-regulation of muscarinic receptor function while regulation of GABA release from CET rats by muscarinic antagonist indicates a down regulation of muscarinic receptor function. These results also indicate decrease in ACh release presynaptically due to up regulation of autoreceptor modulation of ACh release. These results suggested that effects of CET on carbachol and atropine modulation of GABA release may involve multiple subtypes of muscarinic receptors, and these different subtypes of muscarinic receptor may regulation of GABA release in different way. The previous chapter described that M1 muscarinic receptors inhibit GABA release, while M2 muscarinic receptors stimulate GABA release. Thus, the balance between these two types of receptors may determine the activity of GABA interneurons. Therefore, we hypothesize that CET alters the muscarinic receptor subtype function in such a way that facilitating M2 muscarinic function may be up regulated, while the inhibiting M1 muscarinic receptor function may be down regulated.

The primary goal of this study was to determine whether CET selectively alters the sensitivity of different muscarinic receptor subtypes on regulation of GABA release. This was accomplished by measuring the effects of a selective muscarinic receptor agonists and antagonists on ^{3}H-GABA release in sucrose- and ethanol-treated rats followed by a 48 hour withdrawal.

Methods

Animals

Long-Evans rats were treated with ethanol or sucrose diet as described in Chapter 2, general methods. After 28 weeks treatment, blood ethanol levels ranged between 150-200 mg/dl measured at 8 A.M. The average body weight was 812 ± 38 g for CET treated rats and 743 ± 25 g for sucrose treated rats. Animals were treated with ethanol for 28 weeks followed by a 48 hour withdrawal period before decapitation as described in methods. All animals survived after 48 hour withdrawal from 28 weeks ethanol treatment.

Drug treatment

^{3}H-GABA release were measured using an adaptation of a method described in Chapter 3. Superfusion with medium containing different concentrations of receptor agonists and antagonists was started 20 min after S1 (45 min after the start of collection) and continued at a constant level until slices were removed from the chambers. Each concentration was tested on at least duplicate slices; at least three control slices (never exposed to drug) were included in each experiment.

ANOVA was used for all parametric analyses as appropriate followed by Dunnett's multiple comparisons. The distribution of S2/S1 data is normal. All S2/S1 data were statistically analyzed using two way ANOVA tests. Data is represented as mean±SEM with N equal to number of rats.

Results

In these experiments, the sucrose- and ethanol-treated animals were decapitated following a 48 hour withdrawal period. Basal ^{3}H-GABA release from hippocampal slices was stable over the 90 min superfusion period regardless of treatments. This was indicated by the fact that the basal release measured before S2 (PS2) was about 75% of that measured before S1 (PS1) regardless of treatment (Table 9-1). The total tritium was not changed regardless of CET or drug treatment (Table 9-2).

The Effects of Oxotremorine and AF-DX116 on ^{3}H-GABA Release in Hippocampal Slices of Sucrose- and Ethanol-Treated Rats Followed by a 48 Hour Withdrawal Period

In sucrose treated control rats, there was a slight increase in ^{3}H-GABA release caused by 0.01 μM oxotremorine, and a slight decrease in ^{3}H-GABA release caused by 10 μM AF-DX116. When the effects of oxotremorine and AF-DX116 were measured in CET rats, there was a significant increase in GABA release caused by oxotremorine and a significant decrease in GABA release caused by AF-DX116. (Figure 9-1 and Table 9-3). ANOVA revealed a significant interaction between CET and drug treatments (F(2, 35) = 5.95; $p < 0.05$), due to effects of oxotremorine and AF-DX116 in CET rats (F(2, 17) = 13.9; $p < 0.05$), and no effects of oxotremorine or AF-DX116 in sucrose treated rats (F(2,17) =2.58; N.S.).

The Effects of McN-A-343 and Pirezinpine on ^{3}H-GABA Release in Hippocampal Slices of Sucrose- and Ethanol- Treated Rats Followed by a 48 Hour Withdrawal Period

In sucrose treated control rats, there was a significant decrease in ^{3}H-GABA release caused by 1 μM McN-A-343. When the effects of McN-A-343 were measured in CET rats, there were no significant changes in GABA release compared to control slices (Figure 9-2 and Table 9-3). ANOVA revealed a significant interaction between chronic treatment and McN-A-343 ($F(2,24) = 5.97$; $p < 0.05$) which was due to a significant McN-A-343 effect in sucrose treated rats ($F(2,14) = 9.66$; $p< 0.05$), but not in CET treated rats ($F(2,14) = 0.75$; N.S.). There was no significant effect of 1 µM pirenzipine on GABA release in either sucrose or ethanol treated rats (Figure 9-2 and Table 9-3).

Discussion

The effects of CET on M1 and M2 muscarinic receptor regulation of GABA release were studied in these experiments. In CET treated rats, oxotremorine significantly increased and AF-DX116 significantly inhibited ^{3}H-GABA release compared to sucrose fed control rats (Figure 9-1). However, unlike in naive Sprague-Dawley rats, there were not detectable effects of oxotremorine and AF-DX116 on ^{3}H-GABA release in sucrose treated control rats. It is not clear if the reduction in the population of M2 muscarinic receptors due to the high sucrose diet may be responsible for the lack of effect of oxotremorine and AF-DX116 in the rats used in this study compared to the younger Sprague-Dawley rats. As described in chapter 6, chronic sucrose has wide effects on changes in neuronal activity in brain. It has been shown that a high sucrose diet increases sympathetic activity and causes high blood pressure (Gradin et al., 1988; kotchen et al., 1991). Sucrose consumption alters receptor function in brain by decreasing dopamine and 5-HT2 / D2 receptor interactions (see Montgomery & Grottick, 1999). Therefore, sucrose may alter cholinergic function in hippocampus perhaps by suppressing M2 muscarinic function in hippocampus. Therefore, it is necessary to further determine the role of sucrose in this animal model.

The observation that CET significantly enhances the effects of oxotremorine and AF-DX116 may suggest that CET selectively increased the function and the sensitivity of M2 muscarinic receptor regulation of GABA release in hippocampus. These results are consistent with the loss of cholinergic input to hippocampus due to chronic ethanol toxicity which may up regulate cholinergic receptor function in hippocampus, especially the facilitating function of M2 muscarinic receptors to regulate GABA release.

In sucrose treated rats, McN-A-343, the M1 receptor agonist significantly inhibited GABA release, but unlike in naive rats, pirenzipine only slightly increased GABA release. It is not clear whetehr this was due to a change in cholinergic receptor subtypes in the sucrose-control rats as described above or due to the limited sample size in this study. In CET rats, there were no detectable effects of either agonist and antagonist. Thus, unlike the effects of M2 agents, in which both agonist or antagonist indicate an increase in the number or efficacy of M2 muscarinic receptors, these results show that only the M1 muscarinic agonists antagonist indicates a decrease in inhibitory M1 muscarinic receptor efficacy after CET. In summary, the increase of GABA release in hippocampus following CET is due to up regulation of facilitory M2 muscarinic receptors and down regulation inhibitory M1 muscarinic receptors.

Table 9-1. The basal ^{3}H-GABA release in hippocampal slices of sucrose- and ethanol- treated rats followed by a 48 hour withdrawal period was not affected by drug treatment. Release was measured before slices were exposed to each electrical field stimulation (PS1 and PS2). Drug (µM) was introduced prior to S2. The values shown are mean ± SEM of PS2/PS1 ratio (n=5-6 for each group).

PS2/PS1 Ratio

µM	Sucrose	CET
Control	0.72 ± 0.05	0.78 ± 0.04
McN-A-343 (1µM)	0.73 ± 0.04	0.79 ± 0.07
Pirenzipine (1µM)	0.72 ± 0.04	0.81± 0.06
Oxotremorine (0.01 µM)	0.78 ± 0.06	0.71± 0.04
AF-DX 116 (10 µM)	0.74 ± 0.06	0.69 ± 0.05

Table 9-2. The total tissue ^{3}H-GABA from hippocampal slices was not changed regardless of CET or drug treatments. The slices were solubilized after superfusion, and total tritium was determined by calculating the tritium in both tissue and superfusate samples. The values shown are mean ± SEM of total ^{3}H-GABA (n=5-6 for each group).

Total ^{3}H-GABA

µM	Sucrose	CET
Control	165842 ± 12547	135418 ± 10589
McN-A-343 (1µM)	124790 ± 10584	149019 ± 14891
Pirenzipine (1µM)	152289 ± 14867	127481± 13245
Oxotremorine (0.01 µM)	117809 ± 10016	101197± 8976
AF-DX 116 (10 µM)	142147 ± 98101	154212 ± 12419

Table 9-3. Electrically-stimulated ^{3}H-GABA release from hippocampal slices of sucrose- and ethanol- treated rats after a 48 hour withdrawal period was measured in the presence of the selective muscarinic subtype agonists and antagonists, McN-A-343, pirenzipine, oxotremorine and AF-DX116. Drug was included 20 min prior to S2. Values shown are means ± SEM of S2/S1 ratio for N=5-6. * $P < 0.05$ compared to release in the absence of drugs.

S2/S1 Ratio

µM	Sucrose	CET
Control	0.74 ± 0.04	0.75 ± 0.04
McN-A-343 (1µM)	0.52 ± 0.05 *	0.78 ± 0.07
Pirenzipine (1µM)	0.81 ± 0.08	0.89± 0.13
Control	0.71 ± 0.03	0.61± 0.02
Oxotremorine (0.01 µM)	0.75 ± 0.03	0.95± 0.10 *
AF-DX 116 (10 µM)	0.56 ± 0.05	0.41 ± 0.02 *

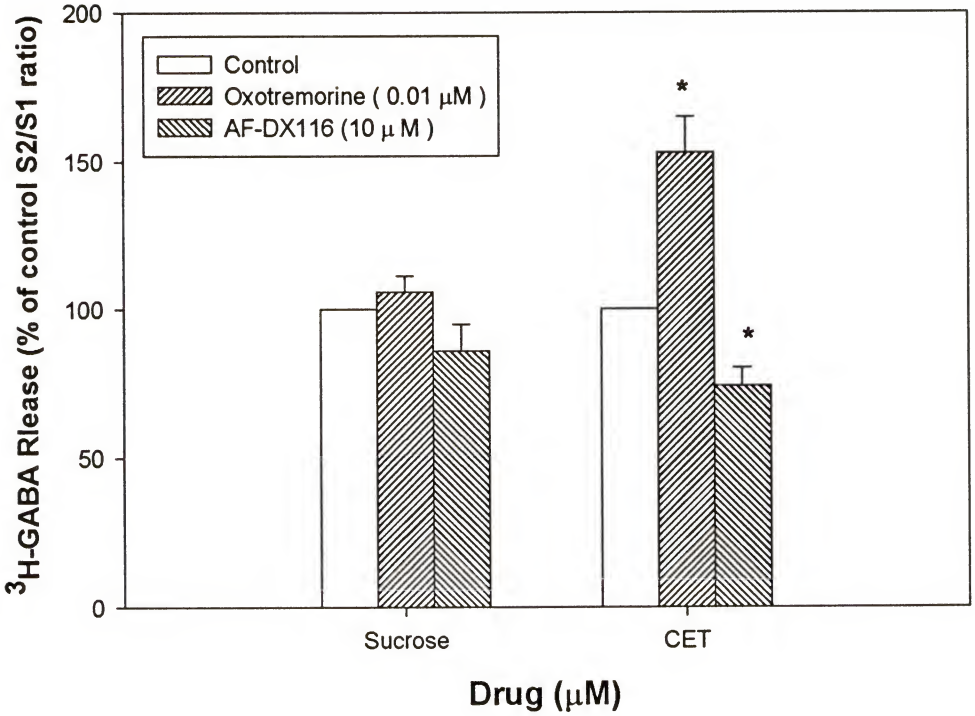

Figure 9-1. The effects of oxotremorine and AF-DX116 on ^{3}H-GABA release in sucrose- and ethanol-treated rats after a 48 hour withdrawal period from ethanol diet. The slices were exposed to 0.01 μM oxotremorine and 10 μM AF-DX116 or control buffer during S2. Oxotremorine significantly increased GABA release in CET rats but not in sucrose treated rats. AF-DX116 significantly inhibited GABA release in CET rats but not in sucrose treated rats. The values shown are means ± SEM for N = 5. * $p < 0.05$ compared to control values.

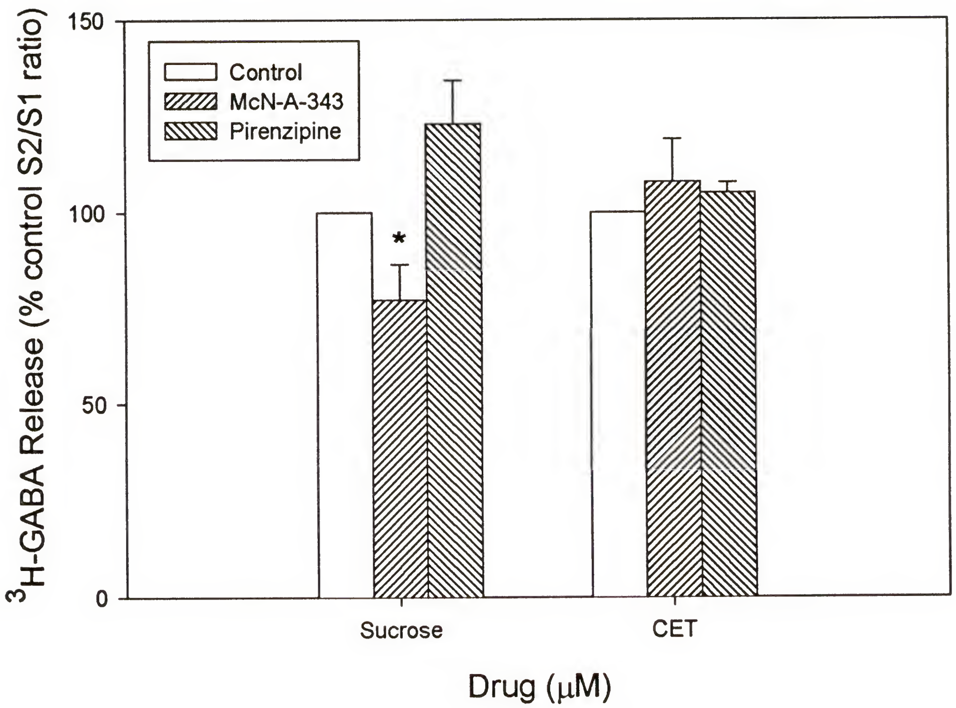

Figure 9-2. The effects of McN-A-343 and pirenzipine on ^{3}H-GABA release in sucrose- and ethanol-treated rats after 48 hour withdrawal from the liquid diet. The slices were exposed to 1 μM McN-A-343 and 1 μM pirenzipine or control buffer during S2. McN-A-343 significantly inhibited GABA release in CET rats but not in sucrose treated rats. There is no effect of pirenzipine on ^{3}H-GABA release in either CET or sucrose treated rats. The values shown are means ± SEM for N = 5. * $p < 0.05$ compared to control values.

CHAPTER 10
SUMMARY AND DISCUSSION

Summary of Results

The main hypothesis tested by the experiments presented in this dissertation was whether long term changes in GABAergic and cholinergic neurotransmitter systems in hippocampus could be the mechanism underlying the inhibition of LTP by CET. Specifically, there are long-term increases in GABAergic neurotransmitter release caused by CET. Different muscarinic receptor subtypes located on GABA interneurons modulated the GABA release in a complicated manner and CET can selectively alter this muscarinic subtype regulation of GABA release. This persistent increase in GABA release could be due to down regulation of inhibitory muscarinic receptors and up regulation of facilitory muscarinic receptors.

In the present studies, we measured the amount of ^{3}H-GABA released from hippocampal slices in response to high frequency electrical stimulation and the modulation of this release by cholinergic presynaptic receptors in adult rat hippocampal slices. The effect of CET on this modulation was also determined. Several observations can be drawn from the results of the experiments performed to test this hypothesis.

1. We established the best stimulation paradigm for eliciting an easily detectable and reliable signal.

2. Our data show that carbachol, a cholinergic agonist, increased GABA release from rat hippocampal slices and the effects of carbachol can be blocked by 10 μM atropine, but not by 10 μM mecamylamine. Thus carbachol is selectively acting at muscarinic receptors in hippocampus to facilitate GABA release.

3. Atropine, when tested alone, modulates GABA release in a biphasic concentration-dependent manner in hippocampus. A possible explanation is that different subtypes of muscarinic receptors may be present in hippocampus and these different subtypes of muscarinic receptors may regulate GABA release in opposite directions. Therefore, the biphasic effects of atropine may be due to its mixed effects on different subtypes of muscarinic receptors.

4. Mecamylamine failed to block the effect of carbachol and mecamylamine alone did not modulate ^{3}H-GABA release. These data indicate that nicotinic receptors are probably not involved in presynaptic regulation of GABA release in hippocampus.

5. The effects of carbachol and atropine were also tested in naive Long-Evens rats and the same results were observed. These data indicate that muscarinic receptors modulate GABA release regardless of different strains of rats.

6. The enhancement of ^{3}H-GABA release by carbachol was significantly increased in slices from CET rats followed by a 48 hour withdrawal period compared to sucrose fed control rat. The biphasic effects of atropine were lessened in similarly-treated rats. These data indicate that different subtypes of muscarinic receptors located on GABAergic neurons may be altered by CET in such

a way that the function of those muscarinic receptors shifted to favor facilitation rather than inhibition of GABA release.

7. The effects of CET on carbachol and atropine were also tested from ethanol treated animals followed by either a 0 hour or 48 hour withdrawal period. CET had the same effect on both animal models regardless of whether withdrawal occurred or not. These results suggest that the changes in presynaptic cholinergic modulation of GABA release are due to neuronal adaption to the 28 weeks of ethanol treatment and are not withdrawal-induced effects.

8. There was no effect of CET on the presynaptic release of ACh. In addition, CET produced no detectable change in presynaptic muscarinic cholinergic autoreceptors which serve to regulate ACh release. Therefore, it appears that the effect of CET is selective for muscarinic receptors located on GABA interneurons, but not those located on terminals of cholinergic neurons in hippocampus.

9. McN-A343, a selective M1 muscarinic receptor agonist, inhibited GABA release and the effects were blocked by pirenzipine, a selective M1 muscarinic receptor antagonist, but not by AF-DX116, a selective M2 muscarinic receptor antagonist. Pirenzipine alone increased GABA release. These results suggest that M1 muscarinic receptors may inhibit GABA release in hippocampus.

10. Oxotremorine, a selective M2 muscarinic receptor agonist, increased GABA release and the effects were blocked by AF-DX116 but not by pirenzipine. AF-DX116 alone inhibited GABA release. These results suggest that M2 muscarinic receptors may facilitate GABA release in hippocampus.

11. Both the effects of oxotremorine and AF-DX116 were significantly enhanced by CET. These results suggest that persistent increases in GABA release may be due to selective up regulation of M2 muscarinic receptor function by CET.

12. The effects of McN-A-343 were decreased by CET. This result suggests that CET may also selectively inhibit M1 muscarinic receptor regulation of GABA release.

Taken together, these results indicate that presynaptic muscarinic cholinergic receptors may play a facilitating role in the regulation of GABA release in the hippocampus, while nicotinic receptors do not exhibit robust regulation of GABA release. CET appears to cause significant changes in this presynaptic regulation of GABA release in hippocampus by increasing facilitating M2 muscarinic receptor modulation of release which is consistent with increased GABA release after CET. This effect is very selective for cholinergic receptors on GABA interneurons as opposed to those on cholinergic neurons. These results are summarized in Figure 10-1. These data may explain how CET increases GABA release from hippocampus, which may contribute to the decrease in LTP seen in rats after CET.

General Discussion

The findings of the present study are that carbachol, a cholinergic agonist, increased GABA release from rat hippocampal slices. Our data show that the effects of carbachol can be blocked by 10 μM atropine, but not by 10 μM mecamylamine. Thus carbachol is acting at muscarinic receptors in hippocampus to facilitate GABA release. These data are consistent with evidence that GABAergic neurons in rat dorsal hippocampus express muscarinic ACh receptors (Walker et al., 1980). Additionally, carbachol causes excitation of hippocampal GABAergic nonpyramidal cells in a direct monosynaptic manner (Pitler & Alger, 1992) and stimulation of cholinergic axons increases GABA activity in hippocampus (Alger, 1991).

Atropine alone modulates GABA release in a biphasic concentration-dependent manner in hippocampus. In brain regions other than the hippocampus, muscarinic receptors sometimes inhibit and sometimes facilitate GABA release (Hajos et al., 1998; Sugita et al., 1991). It is possible that different subtypes of muscarinic receptors may be involved in these different regions and these different subtypes of muscarinic receptors may regulate GABA release in opposite directions. Therefore, the biphasic effects of atropine may be due to its mixed effects on different subtypes of muscarinic receptors.

Our results show that enhancement of ^{3}H-GABA release by carbachol was significantly increased in slices from CET rats compared to sucrose fed control rats. Surprisingly, the effects of atropine were lessened in similarly-treated rats. Thus, unlike the effects of $GABA_B$ agents, in which both agonists and antagonists indicate a decrease in the number or efficacy of autoreceptors (Peris et al., 1997), muscarinic agonists indicate an increase in receptor efficacy while the antagonist indicates a decrease in presynaptic receptor modulation of GABA release.

One possible mechanism is that CET may decrease ACh release presynaptically, which would increase the effect of an exogenously applied agonist but decrease the effect of an exogenous antagonist. However, we did not find an effect of CET on presynaptic release of ACh (Figure 7-1) or on presynaptic muscarinic cholinergic autoreceptor function (Figure 7-2), when ACh release was measured under identical stimulation conditions as GABA release. As described in chapter 7, there could be many reasons for the failure to see an effect of CET on regulation of ACh release presynaptically in our experiments. Therefore, it is still not clear whether CET decreases ACh release presynaptically or not.

However, it is also possible that CET does not alter the presynaptic release of ACh but instead alters muscarinic receptor subtypes that have selective effects on GABA release in hippocampus. In view of these findings, it appears that the effect of CET is selective for muscarinic receptors located on GABA interneurons, but not those located on cholinergic neurons in hippocampus.

Previous studies showed that CET does not alter the muscarinic receptor subtype densities in hippocampus as determined by either immunoprecipitation of m1-5 subtypes (Rothberg et al., 1993) or by maximal ^{3}H-QNB binding or carbachol displacement of specific ^{3}H-QNB binding (Rothberg et al., 1996). Our results are not disproved by these studies, but instead, by measuring the cholinergic regulation of GABA release, we can study the small population of muscarinic receptors located on GABAergic neurons. Our data indicate that only the small population of muscarinic receptors is altered by CET. Thus CET may increase GABA release in hippocampus by both a reduction in presynaptic $GABA_B$ autoreceptors on

GABAergic terminals in hippocampus (Peris et al., 1997) as well as a shift in the function of muscarinic receptors on these GABAergic terminals in hippocampus to increase GABA release.

The animals in our studies were treated with ethanol for 28 weeks followed by either a 0 hour or 48 hour withdrawal period. CET had the same effect on cholinergic modulation of GABA release regardless of whether withdrawal occurred or not. These data indicate that the changes in cholinergic modulation of GABA release are due to neuronal adaptation to the 28 weeks of ethanol treatment and are not withdrawal-induced.

ACh has been shown to increase the population spike amplitude in stratum pyramidale and inhibits EPSPs in stratum radiatum (Rothberg & Hunter, 1991). CET decreases the effects of ACh on population spike amplitude but has no effect on inhibition of EPSPs. The latter effect is thought to be due to the presence of presynaptic muscarinic cholinergic receptors on the terminals of the stratum radiatum afferents thereby reducing neurotransmitter release. The effect on the population spike is thought to be mediated by a pharmacologically distinct set of receptors. Either way, these data do not support our evidence that cholinergic receptor function is up regulated in CET rats. Similarly, both cholinergic disinhibition and recurrent inhibition are decreased by CET (Pitler et al., 1992). Again, it is possible that different subtypes of muscarinic receptors are involved in these processes and these subtypes are affected differently by CET.

Neuronal nicotinic ACh receptors are involved with cognition and have been shown to be potential targets for ethanol effects. Carbachol is not selective for muscarinic or nicotinic ACh receptors, thus, the enhanced effect of carbachol to increase ^{3}H-GABA release may be mediated by nicotinic receptors. However, there is no evidence that nicotinic ACh receptors are present on GABAergic interneurons in the hippocampus. Additionally, our data showed that mecamylamine failed to block the effect of carbachol and mecamylamine alone did not modulate ^{3}H-GABA release. These data indicate that nicotinic receptors are probably not involved in presynaptic regulation of GABA release in hippocampus although more definitive evidence should be obtained by testing the effects of specific nicotinic agonists. Along these lines, CET-induced increases in hippocampal GABA release probably do not involve changes in nicotinic ACh receptor regulation.

Further studies found that oxotremorine, a M2 muscarinic agonist, increased GABA release in a concentration-dependent manner at nM concentrations but not at higher concentrations. Receptor binding studies with oxotremorine indicate the presence of two binding sites with Kd values of 1.2 nM and 446 nM (Miyoshi et al., 1986). Thus, oxotremorine modulates GABA release in an facilitory manner via the high affinity binding site whereas concentrations consistent with occupation of the low-affinity site offset this facilitation. This result may indicate that oxotremorine modulates GABA release by selectively acting at different subtypes of muscarinic receptors.

Some studies have shown that M1 muscarinic receptors may mediate inhibition or facilitate effects on GABA release depending on brain region. In rat cerebral cortex (Hashimoto et al., 1994), M1 muscarinic receptors mediated inhibition of GABA release, while in neostriatum, they facilitated GABA release (Harsing & Zigmond 1998). The effects of M2 muscarinic receptors regulation of GABA release are also regionally dependent. It has been reported that M2 muscarinic receptor mediated stimulation of GABA release in the rat substantia nigra (Kayadjanian et al., 1994). Our data indicate that M1 muscarinic receptors inhibited GABA release, while M2 muscarinic receptor stimulated GABA release in rat hippocampus. Since muscarinic receptors are located on both cell bodies and terminals, it is not clear which population of these muscarinic receptors regulate GABA release. Therefore it is necessary to distinguish the location of these receptors in future studies.

Our data suggested that CET increases GABA release due to up regulation of facilitory M2 muscarinic receptor function. In CET treated rats, oxotremorine significantly increased ^{3}H-GABA release and AF-DX116 significantly inhibited ^{3}H-GABA release compared to sucrose fed control rats. Unlike naive rats, there were no detectable effects of oxotremorine and AF-DX116 in regulation of ^{3}H-GABA release in sucrose treated control rats. It is not clear if the loss of population of M2 muscarinic receptors due to aging in sucrose treated animal may be responsible for the lack of effects of oxotremorine and AF-DX116 in these animals or whether this insensitivity is related to the comsumption of liquid diet. The observation that CET significantly enhances the effects of oxotremorine and AF-DX116 may suggest that CET selectively increases the function and the sensitivity of M2 muscarinic receptors in regulation of GABA release in hippocampus. Perhaps the loss of cholinergic input to hippocampus due to chronic ethanol toxicity up regulates cholinergic receptor function in hippocampus, especially the facilitating function of M2 muscarinic receptors to regulate GABA release.

On the other hand, CET may also alter the function of M1 muscarinic receptors in regulation of GABA release. In sucrose treated rats, the M1 receptor agonist significantly inhibited GABA release, but unlike naive rats, the antagonist failed to increase GABA release. In CET rats, there were no detectable effects of either agonist or antagonist. Thus, unlike the effects of M2 agents, in which both agonist and antagonist indicate a increase in the number or efficacy of M2 muscarinic receptors, these results show that only M1 muscarinic agonists indicate an decrease in receptor efficacy in CET. One possible explanation is that CET may shift the efficacy of M1 receptor to favor agonist. It is should be pointed out that only one concentration of pirenzipine was tested in sucrose and CET rats. It is possible that sucrose and CET treatment may alter the affinity of M1 muscarinic receptors to pirenzipine. Therefore more concentrations of these drugs should be tested on sucrose and CET rats.

Future Directions

The observations of these studies indicated that increased GABA release following CET is likely to be due to up regulation of M2 muscarinic receptors or down regulation of M1 muscarinic receptors or both. It would therefore be of interest to determine if these neuronal adaptions to CET are directly responsible for

CET induced inhibition of LTP. Combined with electrophysiology methods, we can study the effects of these selective muscarinic receptor agonists and antagonists on regulation of GABA release and induction of LTP simultaneously in hippocampal slices from CET rats.

Muscarinic receptors are located on both the cell body and terminals of GABA interneurons, and our data did not show the localization of the muscarinic receptors that regulated GABA release. It is necessary to determine which population of muscarinic receptors mediated the regulation of GABA release and if CET selectively alters this regulation. We can measure the effects of these selective muscarinic receptor agents on regulation of GABA release directly from hippocampal synaptosomes, or in the presence of tetrodotoxin which blocks action potentials, therefore isolating the effects of these muscarinic receptor agents to the synaptic terminals of GABA neurons in hippocampus. Microdialysis techniques which could measure in vivo GABA release from discrete regions might provide further insight to this problem.

Muscarinic receptors are coupled to G-proteins, therefore, besides the receptor itself, the muscarinic receptor-related second messenger systems and effectors may also be a target for CET induced modulation of GABA release. The future studies should be able to use molecular, pharmacological and physiological methods to study the interaction between those effectors and muscarinic receptors in regulation of GABA release in hippocampus and how CET affects this interaction.

A Closing Statement

While it is clear that the studies presented in this thesis represent a small advance in our knowledge of the lasting effects of chronic ethanol, it is equally clear that we are far from a complete understanding of the mechanisms underlying the effects. It is our hope that these studies will stimulate interest in this area of research, and ultimately lead to new treatment strategies which may begin to alleviate the damage and lasting functional deficits associated with chronic alcoholism.

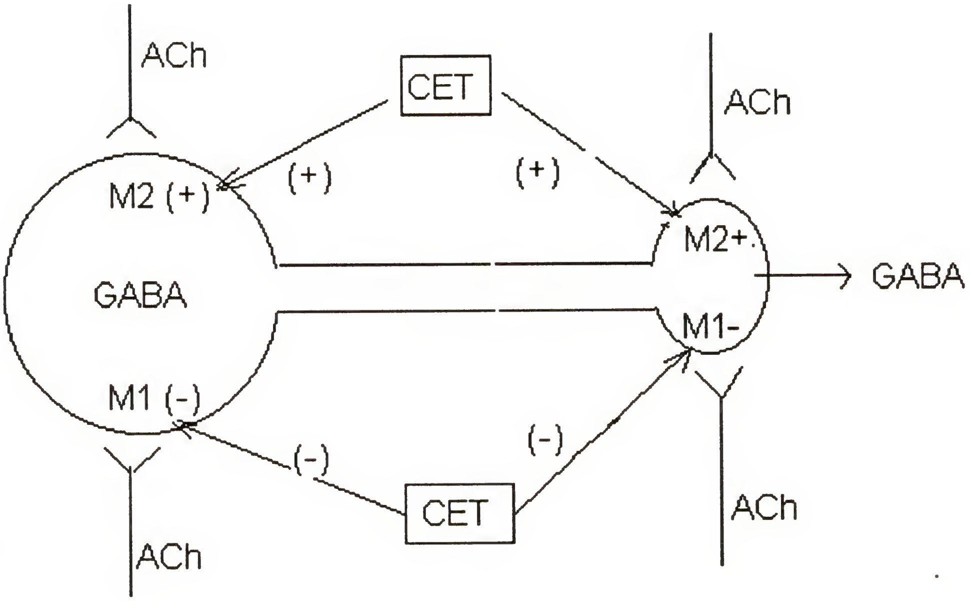

Figure 10-1. Schematic diagram of hypothesized neuronal network in hippocampus. GABAergic interneurons (shown as a circle) are innervated by cholinergic input. Muscarinic receptors are located on both cell body and terminal. ACh inhibits the activity of GABAergic interneurons via M1 muscarinic receptors and facilitates GABAergic interneurons via M2 muscarinic receptors. CET increases GABA release by increasing M2 muscarinic receptor function or by inhibiting M1 muscarinic receptor function.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it confirms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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